Evolution of mutualistic microbiome in firebugs and cotton stainers (Hemiptera; Pyrrhocoridae)

-Characterizing the role of bacterial symbionts in diversification and niche expansion of Pyrrhocoridae

Dissertation
To Fulfill the
Requirements for the Degree of
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…from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved*.*

The Origin of Species, **Charles Darwin**

Table of Contents

LIST OF PUBLICATIONS

This thesis is based on the following manuscripts:

Manuscript I (Chapter 2)

Geographic and ecological stability of the symbiotic mid-gut microbiota in European firebugs,

***Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae)**

Sudakaran S, Salem H, Kost C, Kaltenpoth M

Molecular Ecology (2012)

Summary

In this study the comprehensive characterization of the gut microbiota associated with the European firebug, *P. apterus* revealed that it is composed of four dominant bacterial species Actinobacteria *(Coriobacterium glomerans* and *Gordonibacter sp.*), Firmicutes (*Clostridium sp.*), and Proteobacteria (*Klebsiella sp.*). Further investigation revealed that gut microbiota was quantitatively and qualitatively consistent across firebugs collected from different geographical locations and reared on different diets.

Author contributions

S.S., C.K. and M.K. designed the study. S.S. (100%) performed the experiments and analysed the data on the bacterial community in the different gut regions and across populations and diets. S.S. (50%) and H.S. (50%) analysed the ontogenetic changes in the microbiota. S.S. drafted the manuscript. M.K. and C.K. read and critically commented on the manuscript.

Manuscript II (Chapter 3)

Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae)

Salem H, Kreutzer E, Sudakaran S, Kaltenpoth M

Environmental Microbiology (2013)

Summary

To understand the functional importance of the microbiota to the firebugs a combination of experimental manipulation and community level analysis was used. The experimental removal of the symbionts by egg surface sterilization resulted in significantly higher mortality and reduced growth rates in firebugs. Community level analyses by quantitative PCR of the core microbiota revealed that both the Actinobacterial symbionts (*C. glomerans* and *Gordonibacter sp.*) are vital for the fitness of the host.

Author contributions

H.S. and M.K. designed the study. H.S. (100%) performed the experiments on *P.apterus* and E.K. (100%) performed the experiments on *D. fasciatus*. H.S. (100%) analysed the data on the experimental manipulation and community level analysis of the bacterial community. S.S. (100%) performed the experiment on the bacterial community composition in *D. fasciatus*. H.S. drafted the manuscript. M.K. read and critically commented on the manuscript. All authors amended the manuscript.

Manuscript III (Chapter 4)

Evolutionary transition in symbiotic syndromes enabled diversification of phytophagous insects on an imbalanced diet.

Sudakaran S, Retz F, Kikuchi Y, Kost C, Kaltenpoth M

ISME J (In press)

Summary

In this manuscript the evolution of the Pyrrhocoridae microbiota association was analyzed by reconstructing the Pyrrhocoridae host phylogeny and comprehensive characterization of the gut microbiota of different species of the Pyrrhocoridae family. The acquisition of the core microbiota in the Pyrrhocoridae family coincided with the evolution of their preferred host plants (Malvales), suggesting that the symbionts enabled their hosts to successfully exploit this imbalanced nutritional resource and subsequently diversify in a competition-poor ecological niche.

Author contributions

S.S., C.K. and M.K. designed the study. Y.K provided pyrrhocorid specimens from Japan. S.S. (100%) performed the experiments and analysed the data on the bacterial community in different species of the Pyrrhocoridae. S.S. (50%) and F.R. (50%) reconstructed the pyrrhocorid host phylogeny. S.S. drafted the manuscript. M.K. and C.K. read and critically commented on the manuscript. All authors amended the manuscript.

CHAPTER 1

GENERAL INTRODUCTION

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Symbiotic interactions have played a major role in the evolutionary diversification and expansion into novel ecological niches of many organisms. This thesis addresses the evolution of a consortium of bacterial symbionts associated with the hemipteran insect family, Pyrrhocoridae and their critical role in the expansion of its hosts into a novel ecological niche (Malvales). The following paragraphs provide an overview on symbiotic associations in insects, the factors (biotic and abiotic) that shape and control the microbial composition in insects, the role of microbes in resource acquisition and their contribution towards niche expansion that can allow for diversification and speciation of its host. Finally, a brief introduction into the biology of pyrrhocorid bugs is provided.

1.1 SYMBIOSES: KEY DRIVERS OF NICHE EXPANSION AND EVOLUTIONARY DIVERSIFICATION

Most organisms on our planet do not live in isolation instead have evolved diverse strategies to interact with one or more species for sustainment and subsequent diversification (Thompson 1999). Thus, species interactions are fundamental drivers of novel phenotype and species diversity (Schluter 2000). Most of the major milestones in the diversification of life can be attributed to the appearance of novel species interactions (Margulis and Fester 1991, Szathmary and Smith 1995). In this context, symbioses involving microbes had a major impact on the evolution of most living organisms (Margulis 1998). This is especially evident in the origin of eukaryotic cells, which represents a symbiotic union of a primitive archaebacterium and a mitochondrial ancestor (Dyall *et al* 2004, Gray *et al* 1999, Margulis 1998, Sagan 1967). Even after the evolution

of the eukaryotic cell, symbiotic interaction with microbes has played a critical role in the diversification of numerous lineages (Price 1991). In terrestrial ecosystems, most species critically depend on symbiotic interactions. For example, the symbiotic acquisition of a primitive cyanobacterium with the ability to photosynthesize, which later evolved into the chloroplast, mediated the origin and diversification of plants (Bonen and Doolittle 1975). Later, the emergence of terrestrial plants was facilitated by their symbiotic relationship with mycorrhizal fungi (Newman and Reddell 1987), and the subsequent evolution of herbivorous animals would not have been possible without their nutrition provisioning symbionts (Margulis and Fester 1991). Also in the marine environments, symbiotic interactions play an important role for many prokaryotes and eukaryotes to expand into otherwise inhabitable environments. An example is the intimate association between chemosynthetic bacteria and marine invertebrates. This symbiosis is wide spread in the ocean across various habitats such as deep-sea hydrothermal vents, cold seeps, shallow-water sea grass and coral reef sediments (Cavanaugh *et al* 2006, Dubilier *et al* 2008). These habitats are enriched with reduced compounds such as hydrogen, hydrogen sulfide and methane (Cavanaugh *et al* 1981, Childress *et al* 1986, Hentschel and Felbeck 1993, Petersen *et al* 2011). Chemosynthetic bacteria utilize the energy released during oxidation of these compounds to fix carbon dioxide and methane carbon into organic matter, thus providing their animal host with necessary nutrition (DeChaine and Cavanaugh 2006, Petersen and Dubilier 2009, Stewart *et al* 2005). The symbiotic interaction between chemosynthetic bacterium and marine invertebrates is an evolutionary innovation that enabled them to thrive in extreme environments such as hydrothermal vents and cold seep where photosynthetic byproducts are relatively scarce (Van Dover 2000). These and many other examples demonstrate that symbioses are major contributors towards speciation and evolutionary innovation.

1.2 SYMBIOTIC INTERACTIONS BETWEEN MICROBES AND INSECTS

Among multicellular organisms, insects are one of the most species rich organisms on earth (May 1988). One of the main reasons for their success is that they have diversified into a wide range of food resources (Bourtzis and Miller 2009). Such diverse feeding habits in insects can be attributed to their association with microbial symbionts (Buchner 1965). In this sense, insects are prime candidates to examine the ecological and evolutionary significance of symbiotic interactions.

Microbial symbionts provide a wide range of benefits to their insect hosts (Bourtzis and Miller 2009, Douglas 2006, Feldhaar 2011, Margulis and Fester 1991, Moran and Baumann 2000). The most common and well represented of the symbiotic interactions are the provisioning of nutrients directly to their insects hosts (Dillon and Dillon 2004, Douglas 2006, Margulis and Fester 1991, Moran and Baumann 2000, Sasaki and Ishikawa 1995, Schafer *et al* 1996). In other cases, the microorganisms are known to assist in detoxifying ingested particles (Dowd 1989, Genta *et al* 2006), modify the hydrogen profiles that are important for nestmate recognition (Matsuura 2003), providing defense against parasites or pathogens (Currie *et al* 1999, Currie *et al* 2003, Kaltenpoth *et al* 2005, Kroiss *et al* 2010, Oliver *et al* 2003, Oliver *et al* 2009) and enabling the exploitation of novel food sources (Tsuchida *et al* 2011). Overall such diverse functions provided by the microbial partners are vital for the insects to invade into otherwise inaccessible ecological niches.

Box 1: Different transmission modes of microbial symbionts

Transmission of symbionts between generations is fundamental for the maintenance of the symbiosis. Microbial symbiotic partners of the insects predominantly transfer between generations by either horizontal or vertical transmission.

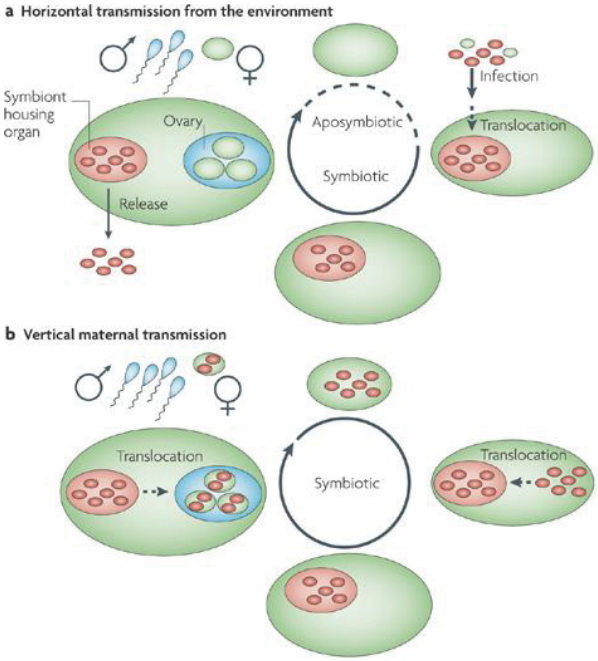

Figure: Different transmission modes of microbial symbionts. From (Bright and Bulgheresi 2010)

Horizontal transmission – In this mode of transmission the insect host produces symbiont free progenies. In every generation these progenies acquire the symbionts from the environment at a specific life stage. In this case free living microbial population serves as inoculum for the symbiosis every generation. In some cases, the symbionts are released by the host to replenish the free living population. The facultative heritable symbionts are predominantly transmitted through this route and some rare cases of gut symbionts.

Vertical transmission – The symbionts are transmitted from mother to offspring most often through germline in vertical transmission. However in some cases, symbionts are also known to transmit vertically in insects through fecal droplets, symbiont containing capsules and egg smearing. Vertically transmitted symbionts are generally obligate intracellular symbionts that have highly reduced genomes but retain metabolic capabilities to supplement beneficial compounds to it hosts.

Mixed mode transmission – A combination of vertical transmission with a low rate of horizontal transmission is known as mixed mode transmission. In general vertical transmission with occasional horizontal acquisition of symbiont from a different population of the same host species or horizontal uptake of symbionts from within the same population but not the parents or from the free living population in the environment constitute for mixed mode transmission. This transmission is traditionally associated with facultative symbiont known for reproductive manipulation of the insect host. However recent findings of this transmission mode with mutualistic symbiosis have started to challenge the traditional view of strict vertical transmission as the favored mode of transmission in insect mutualists.

Symbionts have been traditionally divided into primary and secondary symbionts. The relationship between primary symbionts and insects are often ancient with an estimated age of 30-250 million years (Baumann 2005). These symbionts are predominantly vertically transmitted and mutualistic in nature often by contributing essential nutrients to their insect host (Douglas 2009). In most cases the primary symbionts are harbored in specialized host cells (bacteriocytes) that constitute larger structures called bacteriomes. This type of symbiotic interaction has been predicted to be present in atleast 15% of insects (Baumann 2005, Buchner 1965). In contrast, secondary symbionts are mostly facultative and are of relatively recent evolutionary origin (Dale and Moran 2006) and their prevalence in insects would range from sporadic to fixation in a host species (Gueguen *et al* 2010, Simon *et al* 2003). These facultative symbionts do not necessarily reside in specialized host cells, but could occur extracellularly in the haemocoel and other insect body parts such as the fat body, gut and ovaries but presumably in lower titer levels than the primary symbionts (Dobson *et al* 1999, Moran *et al* 2008). In comparison to the primary symbionts, most secondary facultative symbionts have the capacity to also horizontally transmit to other insect population in addition to vertical transmission (Dale and Moran 2006, Oliver *et al* 2010, Russell *et al* 2003). However the exact frequency of such horizontal transmission events employed by secondary symbionts is relatively unknown. The interaction between facultative symbionts and insects can range from beneficial to parasitic. They are maintained in the insects through several different mechanisms such as providing direct benefits to the insect and thereby being maintained by selection, manipulating the host sex ratio of the insects to produce more females that carry the symbionts and reducing the compatibility of uninfected females to mate with infected males (Hurst and Darby 2009). In rare cases, known secondary symbionts infect insect hosts with high abundance and without any detrimental parasitic behavior but

rather provide direct benefit to the host such as in bed bugs were the well-known reproductive manipulator *Wolbachia* has evolved to supplement B vitamin to the compensate host diet restriction (blood meal) and does not sanction any negative fitness effect on its host (Hosokawa *et al* 2010b). Overall, exceptions like these blur the difference between the primary and secondary symbionts.

Microbes residing in the gut of the insects could be either permanent inhabitants and usually beneficial or transient and occasionally detrimental to the host (Brune and Ohkuma 2011, Moya *et al* 2008). Numerous insects harbor microbes in their gut either in specialized gut compartments such as pouches and caecal invaginations or in the gut lumen (Brune and Ohkuma 2011, Glasgow 1914, Miyamoto 1961). Despite being localized extracellularly, in several cases the symbionts are vertically transmitted via the egg surface, symbiont containing capsules and fecal droplets (Brune and Ohkuma 2011, Kaltenpoth *et al* 2009, Kikuchi *et al* 2009). This has enabled the gut microbiota to coevolve with their insect host. Such specific gut microbiota could play an important role in provisioning nutrients to the host or aiding in digestion of ingested particles such as lignocellulose in termites (Brune and Ohkuma 2011). Finally, insects have also benefitted from forming close relationships with microbes that occur in the close proximity outside their body cavity. These environmental microbial associates are predominantly found in insect induced galls and galleries were they aid in breakdown of complex plant material as well as detoxifying plant allelochemcials (Aylward *et al* 2012, De Fine Licht *et al* 2013). All the major types of microbial symbiotic interaction discussed above are summarized in table 1.

Table 1: Major types of insect–microbe interactions (Adapted from Oliver and Martinez 2014).

Symbiont types	Transmission	Insect host range	Major roles
Obligate intracellular symbionts: Mostly bacteria, some fungi.	Strictly vertical	Present in many hemipteran groups feeding on nutritionally imbalanced phloem, xylem, and vertebrate blood. Also found in carpenter ants, cockroaches, tsetse flies, some Psocodea, and Coleoptera.	Nutrient provisioning, rarely defense
Heritable facultative symbionts: Predominantly intracellular and occasionally extracellular. Mostly bacteria.	Mixed transmission mode : both vertical and horizontal	Widespread across insects, although infections are often sporadic within and among host lineages.	Host protection, reproductive manipulation, and some likely mediate herbivore-plant interactions
Gut microbiota: Extracellular. Mostly bacteria, some fungi and other eukaryotic microbes	Vertical transmission as well as environmentally acquired with varying specificity, but also social transmission	Widespread across insects, but highly variable in terms of microbial diversity. Associations are often more specific and specialized in some groups including xylophages and social insects.	Breakdown cellulose and other plant polymers, nutrient provisioning, nitrogen recycling and fixation, detoxify plant defenses and pesticides, protect against ingested pathogens
Environmental associates: Mutualistic associations between insects and environmental microbes, including plant pathogens that confer benefits to insect vectors. Often fungi, bacteria, and viruses.	Environmental and social	Leaf-cutter ants, sap-feeding Hemiptera, xylophagous insects	Food source, mediate resource acquisition, aid in counteracting plant defenses

1.3 EVOLUTIONARY HISTORY AND ECOLOGICAL FACTORS SHAPE SYMBIONT COMPOSITION

Recent advancements in next generation sequencing technologies have enabled the characterization of symbiotic communities across many insect species (Colman *et al* 2012, Engel and Moran 2013, Jones *et al* 2013, Yun *et al* 2014). This increasing amount of data can aid in elucidating the causes of variation in insect-associated symbiotic communities and their impact within and across phylogenetically related species.

In many insects, ecological factors (such as diet and habitat) have an important influence on the symbiotic gut microbiota and environmental microbial associates. For example, changes in diet during ontogeny are associated with significant variations in microbiota composition of *Drosophila* fruitflies and *Cephalotes* ants (Chandler *et al* 2011, Hu *et al* 2014). Interestingly, a large scale study on the characterization of microbial communities associated with 62 different insect species from various orders highlighted that the microbial composition of distantly related insect taxa feeding on similar diets were highly similar (Colman *et al* 2012). Considering that several other studies have shown that the host associated microbial communities are significantly different from the environmental ones (Duguma *et al* 2013, Hu *et al* 2014), this convergence in symbiont composition could partly have resulted from the adaptation for optimal exploitation of the diet. In order to maintain this microbial community, the host likely evovled filtering processes to prevent establishment of non-native/non-cooperative bacteria. Such a mechanism has been recently shown in the solitary beewolf wasp, where the inoculation of non-native *Amycolatopsis* led to initial colonization but the bacteria were prevented from transmission to the next generation (Kaltenpoth *et al* 2014).

In addition to ecological factors, a collection of host related factors (i.e. phylogenetic signal) such as their evolutionary history, immune effectors and localization within the insect body cavity (e.g. midgut) could also be involved in shaping of the symbiont community (Brucker and Bordenstein 2013, Dietrich *et al* 2014). Several studies have found a phylogenetic correlation between the symbiont composition and its insect host phylogeny. For example, different species of the ant genus *Cephalotes* exhibit species-specific microbial communities although individuals from the same species were collected from different geographic locations and colonies. The existing differences among the microbiota observed across the species significantly correlates with the host phylogeny (Sanders *et al* 2014). Similarly, phylogenetic correlation was also observed between three species belonging to the wasp genus *Nasonia* and its associated microbiota, when all species were reared on the same diet (Brucker and Bordenstein 2012).

While these studies show that both the ecological and host factors could influence the composition of microbial communities associated with insects, they also underline the importance of the host's factors in filtering environmental communities.

1.4 ROLE OF HOST IMMUNITY IN MAINTAINING SYMBIOSIS EQUILIBRIUM

The symbiotic microbiota resides in specific localizations (e.g. gastrointestinal tract) within the insect body cavity. Such symbiont-bearing organs are generally characterized by distinct biotic and abiotic factors (e.g. pH, temperature, oxygen, metabolite, antimicrobial peptides) that are affected by both the host and its symbionts (Buchon *et al* 2013). Recent advances provide a glimpse into mechanisms involved in shaping of the

symbiont residing niche and how the composition and abundance of native microbial communities are regulated.

In the midgut of many insects, the host immune system could play a major role in moderating the persistence and stability of host associated microbiota. For example, In *Drosophila melanogaster*, the immune system not only plays a central role in preventing pathogen infection, it also regulates the resident bacterial population. The intestinal homeobox gene *Caudal* regulates the resident gut microbial community by repressing the nuclear factor kappa B–dependent antimicrobial peptide genes, and silencing the *Caudal* gene using RNAi resulted in the overexpression of antimicrobial peptides, which in turn altered the microbial population in the gut (Ryu *et al* 2008). This process led to the uncontrolled overproliferation of specific resident bacterial taxa, particularly *Gluconobacter morbifer*, resulting in the induction of apoptosis and eventually the demise of the fly (Ryu *et al* 2008).

Alternatively, the intracellular microbial symbionts could be regulated by compartmentalizing them in specialized structures, where a specific immune response is locally triggered. For example, in *Sitophilus* weevils, the primary endosymbiont is confined to the bacteriomes by the localized secretion of an antimicrobial peptide, coleoptericin A, which triggers the formation of giant filamentous structures by the symbionts, which are then unable to spread to other insect cells (Login *et al* 2011).

In many insect species, the immune system has adapted to recognize the beneficial symbiont based on their molecular profiles. In tsetse flies, a peptidoglycan recognition protein with amidase activity (PGRP-LB) is specifically upregulated in the bacteriocytes housing the vitamin supplementing symbiont *Wigglesworthia glossinidia* (Hu and Aksoy 2006). The PGRP-LB functions by degrading the symbiont's peptidoglycan ligand that

initially triggers the immune response, thereby enabling the adaptive persistence of the symbiont in the host (Hu and Aksoy 2006). Alternatively, certain insect systems like aphids have completely lost PGRPs and IMDs, possibly to maintain their relationship with beneficial microbes (*Buchnera*) (Gerardo *et al* 2010).

These studies demonstrate the important role of the insect immune system in preserving the relationship between the insect and its resident microbial community. It also highlights that host-symbiont interactions, similar to associations with parasites and pathogens, play a critical role in the evolution of the immune system in insects.

1.5 SYMBIONT-MEDIATED RESOURCE ACQUISITION

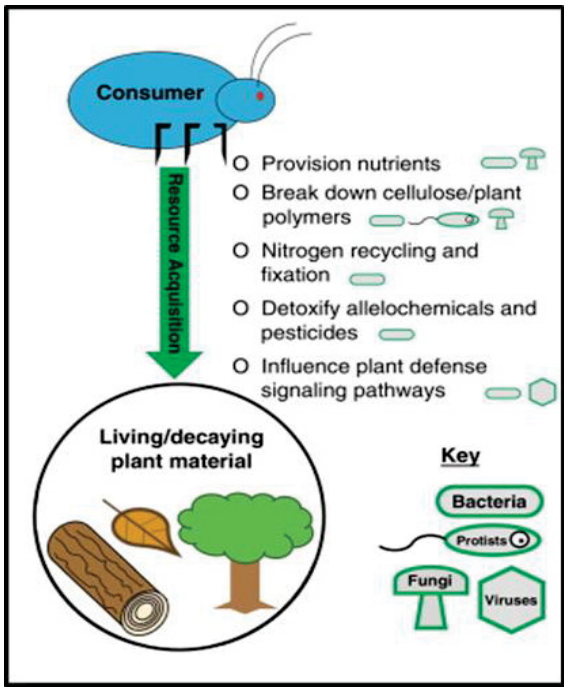

Figure 1: Schematic diagram depicting the numerous beneficial functions provided by the wide diversity of microorganisms associated with insects for resource acquisition. Adapted from (Oliver and Martinez 2014).

The vast diversity of insects observed on earth display phytophagous or xylophagous feeding behavior. There are several obstacles to resource acquisition associated with these feeding behaviors such as low nitrogen content, the availability of essential amino acids, the presence of indigestible components, and the hindrance of chemical defenses (Douglas 2013, Hansen and Moran 2014). In many cases microbial symbionts are relied upon to overcome such obstacles, since they can often provide the necessary nutritional resources for the sustenance of

the insect host (Fig. 1).

One of the major challenges faced by the insects is the variable nitrogen level and the non-uniform availability of essential amino acids across different plant tissues (Douglas 2013, Hansen and Moran 2014). To address this issue, several insect groups have forged a mutually beneficial relationship with nutrient provisioning microbial partners to exploit such nutritionally deficient food sources. These microbial partners are predominantly intracellular symbionts with highly reduced genome but still retain all the necessary biosynthetic genes required for supplementing essential amino acids, vitamins and cofactors (Douglas 2013, Hansen and Moran 2014). In some cases, dual obligate intracellular symbionts are harbored by the insects. In such cases both the symbionts have reduced genomes and complement each other in nutrient provisioning capabilities (McCutcheon and Moran 2010).

Alternatively insects also associate with extracellular microbial partners predominantly localized in the gastrointestinal tract (Engel and Moran 2013). These microbial partners in addition to provisioning nutrients to the host are also capable of performing other diverse roles such as breakdown of indigestible products like lignocellulose, recycle nitrogenous waste, fix atmospheric nitrogen, and detoxify plant allelochemicals (Oliver and Martinez 2014). The most extensively studied example in this regard is the termite-gut microbiota symbiosis. Termites are specialized xylophagous feeders and thus encounter the problem of breaking down ingested polysaccharides such as lignocellulose, which is indigestible by the insect itself. Hence mutualistic association with microbial partners harbored in the hind gut has enabled termites to efficiently digest such recalcitrant plant polymers (Brune 2014, Fig. 2). The gut microbiota of the termites in addition to aiding in the digestion of plant fibres also play an role in provisioning nutrients to the termite host. Lignocellulose, the main component of the termite diet contains very low nitrogen and negligible amounts

of vitamins and aminoacids. In response the microbial partners of the termites compensate for the diet deficiency by recycling nitrogenous waste of the termites and assimilate them into ammonia, which is metabolized into amino acids and vitamins that are then utilized by the host (Abe *et al* 2000, Brune 2014). The diversity of *nifH* genes and the presence of numerous nitrogen fixing strains in the termite gut indicate they could be involved in fixing atmospheric nitrogen to amend the nitrogen budget in the termite host (Hongoh *et al* 2008, Yamada *et al* 2007). Overall, the microorganisms in the hindgut of termites are an essential component in the digestion of lignocellulose; in addition they are also involved in supplementing nutrients through nitrogen recycling and dinitrogen fixation.

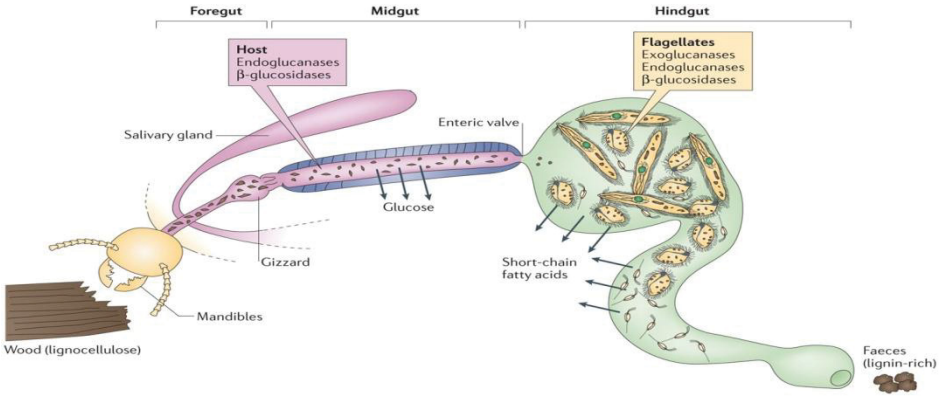

Figure 2: The effective digestion of lignocellulose, an abundant plant polymer by the combined activity of host and gut microbiota. The cellulose digestion is performed by flagellates in lower termites and replaced by hindgut bacteria in higher termites. From (Brune 2014).

Plants have evolved a range of strategies from emitting volatile repellents to toxic secondary metabolites to protect against herbivore attacks. In response insects have evolved behavioral and intrinsic physiological adaptations that counteract the plant defensive allelochemicals. In addition to insects' adaptations, few recent studies have shown that microbial symbionts could also assist in detoxifying plant's chemical defenses. For example, several bacterial symbionts residing in the gut of mountain pine

beetle *Dendroctonus ponderosae* encode for terpene detoxifying biosynthetic pathways (Adams *et al* 2013). Terpenes are a widespread chemical defense used by conifers such as pine and spruce to reduce herbivore attacks (Adams *et al* 2013). Another example is laccase enzyme produced by the symbiotic fungus *Leucocoprinus* associated with leaf cutter ants. The laccase enzyme is consumed by the ant, passes through its gut and is deposited on the top of the fungus garden where it breaks down toxic phenolic compounds from the freshly collected leaves (De Fine Licht *et al* 2013).

In rare cases, symbiotic microbes have also been employed to modulate plant signaling pathways responsible for reducing herbivory attacks. One such case is the Colorado potato beetle, *Leptinotarsa lycopersicum*, which uses orally secreted bacteria to activate antibacterial salicylic acid to interfere with the induction of jasmonic acid resulting in the inhibition of plant defenses (Chung *et al* 2013). Additionally, a few studies have also indicated that several plant pathogens vectored by insects, are in turn beneficial to the insect hosts. For example, a viral pathogen vectored by the whitefly, *Bemisia tabaci*, suppresses plant defenses and terpene production in tobacco (Luan *et al* 2013). Furthermore in tomato, the psyllids, *Bactericera cockerelli,* is able to suppress herbivore-related plant defenses when vectoring *Liberibacter psyllarous*, but not when devoid of this plant pathogen (Casteel *et al* 2012).

1.6 DARWIN'S BLIND SPOT: SYMBIOSIS FACILITATES SPECIATION AND DIVERSIFICATION IN INSECTS

While symbionts are known to provide a multitude of beneficial functions to the insect host, studies that link variations observed in insect microbiota within and between related species to different phenotypic traits, which are potentially involved in speciation and diversification of insects are scarce.

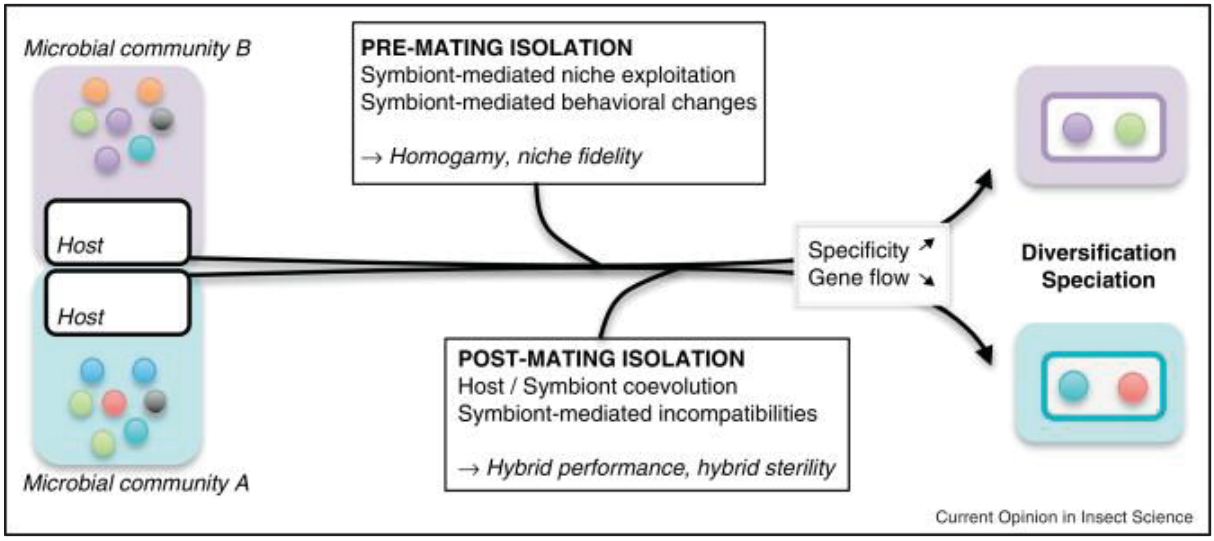

Figure 3: Model indicating various pre-mating and post-mating isolation mechanisms mediated by the microbial symbionts that can facilitate the diversification of the insect host population. From (Vavre and Kremer 2014).

The first category relates to phenotypic traits induced by post-mating isolation mechanisms. In this mechanism the symbionts can interrupt gene flow between conspecific individuals harboring different microbiota resulting in host reproductive isolation and, subsequently, speciation (Vavre and Kremer 2014, Fig. 3). Both vertically and horizontally transmitted symbionts can be involved in post mating isolation by inducing sterility. In *D. paulistorum* and *Nasonia* species, sterility is caused by uncontrolled proliferation of symbionts (*Wolbachia*) in the testes of hybrids (Chafee *et al* 2011, Miller *et al* 2010). Although the exact mechanisms involved in the control of the

symbiont population in hybrids are not yet known, it is highly likely that genetic cross-talk between host and symbiont is essential for the regulation of the symbiont population. This is supported by the findings in *Nasonia* species, in which the hybrid's viability was restored by the removal of the gut microbiota (Brucker and Bordenstein 2013). Inviable hybrids showed an altered gut microbiota and a strong melanization reaction coupled with the activation of the host's immune system. This suggests that the mechanisms involved in the regulation of the symbiont population in *Nasonia* rapidly diverged between closely related species and resulted in post-zygotic barriers to hybrid formation (Brucker and Bordenstein 2013). In addition, microbe-microbe interactions could also influence post mating isolation. The best studied case in this regard is the hybrid mortality induced in *Drosophila* by bidirectional incompatibility in crosses between individuals infected with different *Wolbachia* strains (Engelstadter and Hurst 2009, Serbus *et al* 2008).

On the other hand, pre-mating isolation could be initiated by the acquisition of novel symbionts which facilitates rapid shifts in ecological niches that were previously inaccessible (Fig. 3). For example, the experimental exchange of *Ishikawaella* symbionts between *Megacopta punctatissima* and *M. cribraria* resulted in the complete switch of host plant utilization, i.e. soybean and pea, respectively (Hosokawa *et al* 2007). In the western corn rootworm *Diabrotica virgifera*, crop rotation (corn versus soybean) used for pest control resulted in a shift in microbiota composition with an increase in *Klebsiella* sp. and *Stenotrophomonas* sp.. This shift resulted in an upregulation of cysteine protease activity that enabled the host to circumvent the anti-herbivory defense of the new host plant (soybean) (Chu *et al* 2013). Both cases demonstrate an influence of microbial symbionts on the adaptation to novel environments, which can ultimately result in speciation and/or diversification. Additionally pre-mating isolation could also be induced by symbiont mediated behavioral changes such as individuals harboring similar symbiotic

communities could exhibit kin recognition and mate preferentially (Lize *et al* 2014, Miller *et al* 2010, Sharon *et al* 2010). For example, the rearing of *Drosophila melanogaster* on different diets resulted in a shift in microbiota composition and positive assortative mating. In particular, the presence or absence of *Lactobacillus plantarum* was found to influence the mating preference of the flies (Sharon *et al* 2010). In the *Drosophila paulistorum* species complex, the *Wolbachia* symbiont might modify the pheromonal profiles of the host by interfering with the synthesis of cuticular hydrocarbons and thereby influence the mating patterns of male and female flies (Miller *et al* 2010).

In summary, symbiosis can facilitate insect diversification through various mechanisms. In such cases, the symbionts provide new physiological capabilities that can enable the insect host to expand into novel ecological niches, a first step towards adaptive radiation. This can inadvertently trigger the diversification of the host. Such diversification of the host could initially be facilitated by the reproductive isolation of individuals harboring the same microbiota. Followed by the resultant rapid evolution of host genes involved in the local adaptation to enable the exploitation of a new ecological niche as well as adapting to the symbiotic partner (immune and developmental genes) may result in the emergence of post-zygotic barriers and increased host-symbiont specificity, thus inducing niche specialization and rapid host diversification (Fig. 3).

1.7 THE PYRRHOCORIDAE – BACTERIAL SYMBIOSIS

1.7.1 Stinkbugs and their microbial symbionts

The infraorder Pentatomomorpha (i.e. the '*stinkbugs*') is one of the most diverse groups in the hemipteran insect order containing over 12,500 species (Henry 1997, Schaefer 1993, Schuh and Slater 1995). All of the taxa in this infraorder are terrestrial and mostly plant feeding, with a few mycophagous (Aradoidea) and predacious (Pentatomidae, Asopinae) taxa (Yao *et al* 2012). Many of these bugs are economically important in agriculture and forestry as they are pest of crops (Schaefer and Panizzi 2000).

Most of the pentatmomorphan bugs harbour beneficial symbionts that contribute significantly to the fitness of their host (Abe *et al* 1995, Fukatsu and Hosokawa 2002, Kikuchi *et al* 2009, Tada *et al* 2011). Within Pentatomomorpha infraorder the most common symbiotic syndrome (i.e. identity and localization of the symbionts) is the specialised sacs or tubular outgrowths, called crypts or gastric caeca, in the posterior region of the mid-gut that harbour beneficial proteobacterial symbionts (Buchner 1965, Glasgow 1914, Miyamoto 1961). Among the pentatomomorphan superfamilies, Lygaeoidea, Coreoidea, and Pentatomoidea harbour Beta- or Gamma- Proteobacteria in such specialised crypt like structures in their midgut (Buchner 1965, Fukatsu and Hosokawa 2002, Glasgow 1914, Hosokawa *et al* 2010a, Kikuchi *et al* 2011, Miyamoto 1961). In addition, several other symbiotic syndromes were characterised in the infraorder, such as paired or unpaired bacteriomes with intracellular symbionts in some Lygaeoidea (Kuechler *et al* 2012, Matsuura *et al* 2012), as well as gut symbionts in a particular mid-gut region of *Pyrrhocoris apterus* (Pyrrhocoridae) (Haas and König 1987, Kaltenpoth *et al* 2009). Overall, the transition between these varied symbiotic syndromes must have occurred repeatedly in the evolutionary history of the pentatomomorphan bugs.

However, the evolutionary significance of such transitions of the symbiotic syndromes and their impact on the functionality of the symbiosis remain largely unknown.

1.7.2 The family Pyrrhocoridae and its gut symbionts

The Pyrrhocoridae family comprises nearly 300 species of terrestrial bugs and are spread across both the Old and New World (Ahmad and Schaefer 1987). Most members of the family are phytophagous and show a clear preference towards four plant families (Malvaceae, Sterculiaceae, Bombacaceae and Tiliaceae) belonging to the Malvales order (Ahmad and Schaefer 1987). Although the bugs are known to aggregate on different parts of the host plant, they feed only on the ripe or ripening seeds, to which the bugs seemed to be attracted by volatile compounds (Ahmad and Schaefer 1987, Kristenová *et al* 2011).

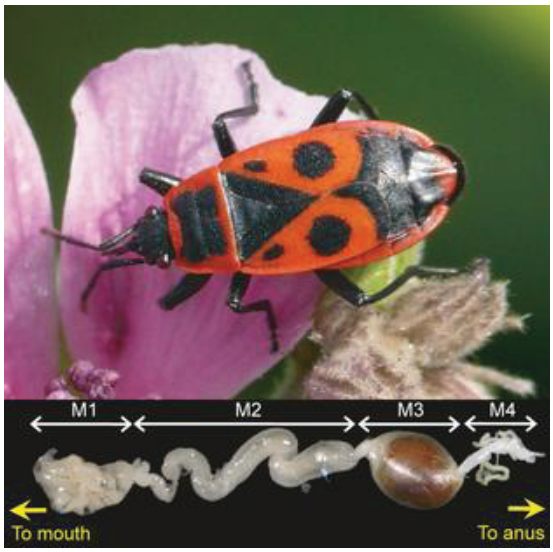

Figure 4 The European firebug (*P. apterus*) (top) and the midgut structure of P. apterus (bottom). The midgut consists of four distinct regions (M1, M2, M3 and M4). The M3 region harbors the gut microbiota.

Among the Pyrrhocoridae family, one of the most common species is the red firebug (*Pyrrhocoris apterus)*(Fig. 4 top), which is widespread across the Palaearctic region. It has been extensively studied in the fields of ecological, biochemical, physiological and endocrinological research (Socha 1993). *P. apterus* predominantly utilizes dry ripe seeds of linden trees (*Tilia cordata* and *T. platyphyllos*) as a food source. However, during limited access to linden seeds, *P. apterus* has been shown to readily exploit seeds of other

Malvales (Kristenová *et al* 2011) and occasionally prey on dead insects, as well as on freshly moulted conspecifics (Henrici 1938, Southwood and Leston 1959).

Previous studies on *P. apterus* reported a specific actinobacterial symbiont (*Coriobacterium glomerans*) localised in the midgut M3 region (Haas and König 1987, Fig. 4 bottom), which is vertically transmitted across generations through egg smearing (Kaltenpoth *et al* 2009). In *P. apterus* and *Dysdercus fasciatus*, the midgut crypts that are commonly found to harbour bacterial symbionts in other pentatomomorphan bugs are reduced in size and do not contain any symbiotic microbes (Buchner 1965, Glasgow 1914). A similar reduction in crypt size is also observed in other Pyrrhocoridae genera *Antilochus* and *Probergrothius* (Goel and Chatterjee 2003, Rastogi 1964, Singh and Singh 2001), indicating that the M3-associated microbiota may be widespread among Pyrrhocoridae. Concordantly, studies in *Dysdercus peruvianus* and *P. apterus* have shown that the ingested food particles are retained for the longest duration in the midgut M3 region, indicating that it is main region for the digestion of ingested food particles (Kodrík *et al* 2012, Silva and Terra 1994). Interestingly, in comparison to other well-known insect-associated symbiotic microbes, *C. glomerans* is not enclosed in specialised symbiont organs, but instead they are localised in the midgut M3 region which is exposed to constant influx of food particles and other transient microbes. Therefore, it is highly likely that other bacterial taxa could co-occur with *C. glomerans* in the M3 region, similar to termites which harbour a complex symbiotic gut microbiota to aid with the digestion of ingested food material. Supporting this hypothesis, several other bacterial taxa (*Streptococcus lactis*, unclassified *Streptococcus,* and *Hafnia alvei*) were previously isolated from the midgut M3 region in the *P. apterus* by Haas and Konig (1987). However, these bacterial taxa were not consistently isolated from the bugs throughout their life.

Within the Pentatomomorpha infraorder, the Pyrrhocoridae appear to be exceptional with regards to both the localisation and identity of the symbiont, making them an interesting group to study the impact of evolutionary transitions in symbiotic syndromes on shifts in ecological niches and, possibly, adaptive radiation of the hosts. The recent advancement in next generation sequencing technologies provides cost- and labour-efficient tools to comprehensively characterise the complex microbial communities associated with insects and allowed for addressing the following questions regarding the Pyrrhocoridae symbiosis: (i) Does *P. apterus* harbour additional symbionts to *C. glomerans*, and if so, are they stable and specific? (ii) Are these gut symbionts beneficial to the pyrrhocorid host? (iii) How widespread are the gut symbionts within the Pyrrhocoridae, did they coevolve with their hosts, and how old is this symbiosis? (iv) And finally the ultimate goal is to understand the impact of the shift in symbiotic syndromes on the switch of pyrrhocorids to Malvales plants and their subsequent diversifcation.

1.8 THESIS OUTLINE

This doctoral thesis aimed to (1) comprehensively characterize the microbiota associated with firebugs and evaluate the stability of the microbiota across different geographical and ecological conditions, (2) assess the specificity of the symbiosis and its impact on host fitness, and (3) elucidate the evolutionary origin of the symbiotic microbiota in the Pyrrhocoroidea superfamily and test if the microbiota coevolved with its hosts.

Chapter 2 describes the characterization of the complex gut microbiota associated with European firebugs (*P. apterus*) and assesses if the gut microbiota is stable across firebugs collected from different geographical locations and reared on three different diets. The continuity of the symbiotic bacterial community across different developmental stages of the firebug was analyzed to assess the population dynamics of the symbionts within the host.

In chapter 3, the combination of experimental manipulation and community level analysis of the gut symbionts was used to demonstrate the importance of the microbiota for the fitness of the host. Additionally, the specificity of the host-microbiota association was tested through reciprocal cross infection of the microbiota between two pyrrhocorid species.

In chapter 4, coevolution between the pyrrhocorid hosts and the symbionts at community and strain level was tested by characterizing the symbiotic gut microbiota of different species of Pyrrhocoridae and reconstructing the pyrrhocorid host phylogeny. Further, the evolutionary origin of the Pyrrhocoridae microbiota symbiosis was dated to check whether it coincides with the evolution of the pyrrhocorid's host plant. Finally the effect of a major transition in the symbiotic syndromes on the Pyrrhcorid bugs ability to exploit Malvales plants is discussed.

Lastly, in chapter 5, we discuss the findings of this thesis in the context of the ecological and evolutionary implications of major transitions in symbiotic syndromes within the hemipteran insect order. Throughout this chapter, we summarize the diverse symbiotic syndromes present across the hemipteran order and expand on the impact of the transition between different symbiotic syndromes from the perspective of the host phylogeny. In addition, the implications of a major transition to a symbiotic gut microbiota in Pyrrhocoridae in a broader perspective within the hemipteran order are discussed.

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CHAPTER 2

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Geographic and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae)

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2.1 ABSTRACT

Symbiotic bacteria often play an essential nutritional role for insects, thereby allowing them to exploit novel food sources and expand into otherwise inaccessible ecological niches. Although many insects are inhabited by complex microbial communities, most studies on insect mutualists so far have focused on single endosymbionts and their interactions with the host. Here we provide a comprehensive characterization of the gut microbiota of the red firebug (*Pyrrhocoris apterus,* Hemiptera, Pyrrhocoridae), a model organism for physiological and endocrinological research. A combination of several culture-independent techniques (454-pyrosequencing, quantitative PCR and cloning/sequencing) revealed a diverse community of likely transient bacterial taxa in the mid-gut regions M1, M2 and M4. However, the completely anoxic M3 region harbored a distinct microbiota consisting of facultative and obligate anaerobes including Actinobacteria *(Coriobacterium glomerans* and *Gordonibacter sp.*), Firmicutes (*Clostridium sp.* and *Lactococcus lactis),* and Proteobacteria (*Klebsiella sp.,* and a previously undescribed Rickettsiales bacterium). Characterization of the M3 microbiota

in different life stages of *P. apterus* indicated that the symbiotic bacterial community is transmitted vertically and becomes well defined between the second and third nymphal instar, which coincides with the initiation of feeding. Comparing the midgut M3 microbial communities of *P. apterus* individuals from five different populations and after feeding on three different diets revealed that the community composition is qualitatively and quantitatively very stable, with the six predominant taxa being consistently abundant. Our findings suggest that the firebug mid-gut microbiota constitutes a functionally important and possibly coevolved symbiotic community.

2.2 INTRODUCTION

Symbiotic interactions with microorganisms play an important role for a broad range of plants and animals (Buchner 1965; Moran *et al.* 2008; Moya *et al.* 2008; Smith 1989). Insects harbour a particularly large diversity of symbiotic microorganisms (Buchner 1965; Moran *et al.* 2008), and in many cases the microbial cells outnumber the host's own cells (Dillon and Dillon 2004). Microbial symbionts have been shown to assist their insect hosts in various functions such as nutritional upgrading of the diet (Akman *et al.* 2002; Douglas 1998), detoxifying the ingested food material (Cardoza *et al.* 2006; Dowd 1989; Genta *et al.* 2006), providing defence against parasites or pathogens (Currie *et al.* 1999, 2003a,; Kaltenpoth 2009; Kaltenpoth *et al.* 2005; Kroiss *et al.* 2010; Oliver *et al.* 2003, 2009), mediating thermal tolerance to the host (Dunbar *et al.* 2007), and facilitating the exploitation of novel host plants (Tsuchida *et al.* 2011). These associations with bacteria can be vital for insects to invade otherwise inaccessible ecological niches (Feldhaar 2011).

Most studies on insect-symbiont interactions so far have focused on individual obligate endosymbionts that are typically harboured in specialised organs, so-called bacteriomes. However, many insects are inhabited by a complex microbial community (Dillon and Dillon 2004; Lysenko 1985), but functional analyses of complete communities are scarce. This is mainly due to problems in discriminating the 'core' indigenous microbial community from more transient microbes that were taken up with the food material as well as the difficulty to specifically manipulate the often very complex communities.

Until recently, detailed culture-independent surveys on insect-associated microbial communities could only be achieved by using polymerase chain reaction (PCR) amplification, cloning, and sequencing of the 16S ribosomal RNA gene, which is time-consuming and costly (Konstantinidis and Tiedje 2005; Wintzingerode *et al.* 1997).

However, recent advances in high-throughput next generation sequencing technologies such as 454 pyrosequencing and Illumina sequencing provide more cost- and labour-efficient alternatives to comprehensively characterize complex microbial communities of environmental samples including insects (Metzker 2010; Sun *et al.* 2011).

The Heteroptera, also known as true bugs, comprise around 38,000 species worldwide, representing one of the most diverse hemimetabolous insect taxa (Henry 1997; Schaefer 1993; Schuh and Slater 1995). The infraorder Pentatomomorpha comprising over 12,500 insect species ("stinkbugs") are predominantly phytophagus species that exploit resources from roots to seeds of their host plants, with the exception of a few predacious and mycophagous species (Henry 1997; Schaefer 1993; Schuh and Slater 1995). It forms a monophyletic clade within the Heteroptera, consisting of five superfamilies Lygaeoidea, Coreoidea, Pyrrhocoroidea, Pentatomoidea, and Aradoidea (Schuh and Slater 1995). Most of the pentatomomorphan bugs have a specific symbiotic interaction with bacteria harboured in sacs or tubular outgrowths, called crypts or caeca, in a posterior region of the mid-gut (Buchner 1965; Fukatsu and Hosokawa 2002; Glasgow 1914; Hosokawa *et al.* 2010a; Kikuchi *et al.* 2011a; Miyamoto 1961; Prado and Almeida 2009). Most of these gut symbionts are vertically transmitted by post-hatching transmission mechanisms such as egg surface contamination, coprophagy, or the formation and deposition of special symbiont-containing capsules by the mother (Abe *et al.* 1995; Hosokawa *et al.* 2005; Kikuchi *et al.* 2009; Prado *et al.* 2006; Schorr 1957). In some cases, experimental elimination of the symbiotic bacteria has resulted in high mortality and reduced growth, indicating that the symbionts of these bugs play an important role for the fitness of the host insect (Abe *et al.* 1995; Fukatsu and Hosokawa 2002; Huber-Schneider 1957; Kikuchi *et al.* 2009; Muller 1956; Schorr 1957; Tada *et al.* 2011).

The Pyrrhocoridae are a family of around 300 species of terrestrial bugs, most of which feed on seeds of plants of the order Malvales. Among them, the red firebug (*Pyrrhocoris apterus)* is one of the most common and widespread Palaearctic species, which has been extensively studied in the fields of ecological, biochemical, physiological and endocrinological research (Socha 1993). Before reaching adulthood *P. apterus* passes through five nymphal instar stages. The development of the initial four instar stages takes approximately 14 days, and they remain between 7 to 10 days in the final instar stages (Socha 1993). Earlier observations suggest that the bugs do not start to feed until they have reached second instar (Puchkov 1974).

P. apterus predominantly utilizes dry ripe seeds of linden trees (*Tilia cordata* and *T. platyphyllos*) as a food source. However, during limited access to linden seeds, *P. apterus* has been shown to easily adapt to seeds of other Malvales (Kristenová *et al*. 2011) as well as host plants from different families (Socha 1993; Tischler 1959). Additionally, firebugs are opportunistic scavengers feeding occasionally on dead insects, and they even attack and consume freshly molted conspecifics (Henrici 1938; Southwood and Leston 1959).

The digestion of food material in pyrrhocorids takes between three to four days (Silva and Terra 1994). Previous studies in *Dysdercus peruvianus* and *P. apterus* have shown that the ingested food particles are retained in the M1 region for only about five hours, whereas the passage through M2 and M3 takes approximately 70 - 90 hours, before the ingested food quickly passes through the M4 and the hindgut (Kodrík *et al.* 2012; Silva and Terra 1994). Despite considerable interest in the digestive processes of *P. apterus*, little is known yet on the symbiotic microbial community that inhabits in the different gut regions and its possible contribution to the host's digestion. However, previous studies reported on a specific actinobacterial symbiont (*Coriobacterium glomerans)* that occupies the mid-gut section M3 (Haas and König 1987, 1988). *C. glomerans* is vertically

transmitted to the offspring through egg smearing (Kaltenpoth *et al.* 2009) and appears to be essential for successful development and reproduction of the bugs (Salem *et al.*, submitted).

In the present study, we used a combination of several culture-independent techniques (454 pyrosequencing, cloning/sequencing, quantitative PCR and fluorescence *in-situ* hybridization) to comprehensively characterize the microbial community that inhabits different regions of the mid-gut of *P. apterus*. Furthermore, we analyzed changes in the microbial community in the course of the bugs' development and examined its compositional stability across different populations and upon different diets. The results allow us to draw conclusions on the intimacy of the host-symbiont association and to speculate on a possible coevolutionary history of *P. apterus* and its symbiotic mid-gut microbiota.

2.3 MATERIALS AND METHODS

2.3.1 Rearing conditions and sample collection

For the characterization of the microbial community composition across different gut regions and life stages, a laboratory culture of *P. apterus* was established with individuals collected in Jena, Germany. The bugs were reared in plastic containers (20 x 13 x 12 cm) at a constant temperature of 28˚C under long light conditions (16:8 h light/dark cycles) to keep the insects in reproductively active stage (Saunders 1983, 1987). Insects were supplied with linden seeds (*Tilia cordata* and *T. platyphyllos*) and water *ad libitum.* The linden seeds, water, and the soil provided in the rearing cages were autoclaved to minimize exposure to environmental microbes.

For the survey of the microbiota in the different gut regions of *P. apterus*, six adult individuals derived from the Jena population were killed by freezing at -20˚C for 1 h. Prior to dissection, the animals were immersed in 0.1% sodium dodecyl sulphate (SDS) and then rinsed with sterile de-ionized water in order to remove surface contaminants. The abdomen of the bug was incised on both sides to remove the dorsal cuticle, the gut was collected under sterile de-ionized water, and the different gut regions were separated and placed into individual Eppendorf tubes for DNA extraction.

In order to analyse changes in the microbial community during the development of *P. apterus*, a cohort of firebugs from six egg clutches from different females was established, and six replicates per life stage were collected and surface-sterilized as described above. For the early life stages (egg to 4th instar nymphs), several individuals were pooled for replicate DNA extractions, respectively (eggs: 6 individuals, 1st instar: 3 individuals, 2nd instar: 3 individuals, 3rd instar: 2 individuals, and 4th instar: 2 individuals)

in order to ensure sufficient DNA extraction yields. In all cases, complete animals were used to extract genomic DNA.

To assess variation in the microbial community composition across different geographical locations, *P. apterus* specimens were collected from five different populations in Central Europe: Jena (n=6), Berlin (n=8), Regensburg (n=8), Würzburg (n=8) (all in Germany) and Maria Saal (n=5) (Austria). The M3 sections of the adults' mid-guts were obtained as described above, and DNA was extracted individually. To assess the effect of different diets on the mid-gut microbiota composition, *P. apterus* individuals derived from the Jena population were reared from egg to the adult stage exclusively on one of three different diets: i) linden seeds (*Tilia cordata* and *T. platyphyllos*), which represent their natural diet in the field, ii) sunflower seeds (*Helianthus annuus*), or iii) a carnivorous diet consisting of larvae of the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae), which had been killed by freezing at -20˚C for 1 h. For all three diet treatments, fresh food as well as sterile water was provided twice a week *ad libitum*. The DNA was extracted individually from the midgut M3 region of five specimens for each diet.

Individual extracts of P. apterus from each life stage, population and diet experiment were used for qPCR analyses, and one pooled DNA sample, respectively, from all bugs of each gut region, life stage, population and diet experiment was used for bacterial tag-encoded 454 FLX pyrosequencing (bTEFAP) of 16S rRNA amplicons. To analyze the transient microbial community that firebugs may take up from the diet, linden seeds (*Tilia cordata* and *T. platyphyllos*) were collected from the field (Beutenberg campus, Jena, Germany) and subjected to bTEFAP.

2.3.2 DNA extraction and amplification

Prior to DNA extraction, all samples were submerged in liquid nitrogen and crushed with sterile pestles. DNA was extracted using the MasterPure^{TM} DNA Purification Kit (Epicentre Technologies) according to the manufacturer's instructions. An additional lysozyme incubation step (30 min at 37 °C; 4 µl of 100 mg/ml lysozyme, Sigma-Aldrich, USA) was included prior to proteinase K digestion to break up gram-positive bacterial cells. The successful extraction of the gut microbial DNA from *P. apterus* was verified by using PCR assays with general eubacterial 16S rRNA primers (fD1 and rP2) (Weisburg *et al.* 1991) (Table 1). Subsequently, the extracted DNA was used for 454 pyrosequencing and qPCR assays.

2.3.3 Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) and data analysis

BTEFAP was done by an external service provider (Research and Testing Laboratories, Lubbock, USA) using the 16S rRNA primers Gray28F and Gray519r (Table 1) (Ishak *et al.* 2011; Sun *et al.* 2011) with primers numbered according to their position in *Escherichia coli* 16S rDNA. Generation of the sequencing library was established through one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar high fidelity *Taq* polymerases (Qiagen). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Research and Testing Laboratory (RTL, Lubbock, TX, USA), based upon RTL protocols (http://www.researchandtesting.com). All low quality reads (quality cut-off = 25) and sequences < 200 bp were removed following sequencing, which left between 7,000 - 15,000 sequences per sample for subsequent analysis.

Processing of the high-quality reads was done using Qiime (Caporaso *et al.* 2010b). The sequences were clustered into operational taxonomic units (OTUs) using multiple OTU picking with cdhit (Li and Godzik 2006) and uclust (Edgar 2010) with 97% similarity cut-offs. For the analysis, the samples were grouped into four datasets namely gut regions, life stages, populations, and diets to ensure that OTUs were clustered consistently across samples by the OTU picking method. For each OTU, one representative sequence was extracted (the most abundant) and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso *et al.* 2010a), with the minimum sequence identity percent set to 75%. Taxonomy was assigned using RDP classifier (Wang *et al.* 2007), with a minimum confidence to record assignment set to 0.80. OTU tables were generated describing the occurrence of bacterial phylotypes within each sample (Tables S1-4). The tables were then manually curated by removing low-frequency reads (<0.5% in all samples) and by blasting the representative sequences against the NCBI and RDP databases. Based on the blast results, OTUs with the same genus-level assignments were combined for visualization of the results. The revised OTU table was used to construct heatmaps using the MultiExperiment Viewer (MeV) software (Saeed *et al.* 2003).

2.3.4 PCR amplification, cloning, and sequencing

To compare the microbial community composition as revealed by bTEFAP to the more conventional cloning/sequencing procedure, a pooled mid-gut M3 DNA sample from six *P. apterus* individuals of the Jena population was used for PCR amplification using general eubacterial 16S rRNA primers (fD1 and rP2, see Table 1). PCR amplification was done using a UnoCycler (VWR International GmbH, Belgium) in a total reaction volume of 12.5 µL containing 1 µL of template DNA, 1xPCR buffer (20 mM Tris-HCl, 16 mM

$(NH_4)_2SO_4$, and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 µM dNTPs, 0.8 µM of each primer, and 0.5 U of Taq DNA polymerase (VWR International GmbH, Belgium). Cycle parameters were: 3 min at 94˚C, followed by 35 cycles of 94˚C for 40 s, 68˚C for 40 s and 72˚C for 40 s, and a final extension step of 4 min at 72˚C. PCR products were cloned using the StrataClone PCR Cloning Kit (Agilent Technologies, USA) according to the manufacturer's instructions. Transformed *E. coli* cells were grown on LB agar containing 10 mg mL^{-1} ampicillin and appended with 2% 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) (Sigma Aldrich, Germany) for blue/white screening. Colony PCR was performed on randomly selected transformants with vector primers M13F and M13R (Table 1) using the above-mentioned reaction mix and cycling conditions except that an annealing temperature of 55°C was used. PCR products were checked for the expected size on a 1.5% agarose gel (130 V, 30 min) and purified using the peqGOLD MicroSpin Cycle Pure Kit (Peqlab Biotechnologies GmbH, Germany) prior to sequencing. In total, 179 clones were sequenced bidirectionally on an ABI 3730xl capillary DNA sequencer (Applied Biosystems, USA) using the M13 primers (Boutin-Ganache *et al.* 2001).

2.3.5 Molecular phylogenetic analysis

Nearly full-length 16S rRNA sequences (1.4kb, obtained from cloning/sequencing) of the six consistently detected microbial taxa in the *P. apterus* M3 mid-gut region were aligned to reference sequences obtained from the Ribosomal Database Project (RDP) using the ClustalW algorithm implemented in MEGA5 (Tamura *et al.* 2011; Thompson *et al.* 1994). Phylogenetic trees were computed using maximum likelihood (Tamura-Nei model, G+I rate variation) with 500 bootstrap replicates in MEGA5 (Tamura *et al.* 2011).

2.3.6 Quantitative PCR

Specific primers for quantitative PCR (qPCR) were designed using Primer3 (http://primer3.sourceforge.net/) for the six most consistently found and abundant bacterial taxa in the M3 region of the mid-gut (*Coriobacterium glomerans*, *Gordonibacter sp.*, *Clostridium sp.*, unknown Rickettsiales *sp.*, *Lactococcus lactis* and *Klebsiella sp.)* based on an alignment of the representative set of sequence data for all OTUs available from the cloning/sequencing and 454 pyrosequencing. PCR conditions for each primer pair were optimized using gradient PCRs with a pooled *P. apterus* gut sample (Jena) as a template (Table 1). The specificity of the primers was verified *in silico* against the RDP database (Maidak *et al.* 2001) and *in vitro* by sequencing the optimized PCR product directly without prior cloning. If the sequence matched the expected OTU, the primer pair was assumed to specifically amplify the target OTU within the *P. apterus* gut. Additionally, specificity was monitored via qPCRs by including a melting curve step at the end to ensure that amplicons were the same across samples for each primer assay.

Quantitative PCRs for individual bacterial symbionts were performed on a RotorgeneQ cycler (Qiagen, Germany) in final reaction volumes of 25 µL containing 1 µL of template DNA (usually a 1:10 dilution of the original DNA extract), 2.5 µL of each primer (10 µM), and 12.5 µL of SYBR Green Mix (Rotor-Gene SYBR Green kit, Qiagen). Standard curves were established by using 10^{-8} – 10^{-2} ng of specific PCR product as templates for the qPCR. A NanoDropTM1000 spectrophotometer (Peqlab Biotechnology Limited, Germany) was used to measure DNA concentrations for the templates of the standard curve. Six different replicates of the standard concentrations for each bacterial taxon were used to calculate a correction factor to alleviate any errors of the template standard curve (e.g. inaccuracies of the DNA concentration measurements). PCR conditions were as

follows: 95˚C for 5 min, followed by 35 cycles of 60˚C for 30 s, 72˚C for 20 s, and 95˚C for 15 s; then a melting curve analysis was performed by increasing the temperature from 60˚C to 95˚C within 20 min. Based on the standard curves, the 16S copy number could be calculated for each individual bug from the qPCR threshold values (Ct) by the absolute quantification method (Lee *et al.* 2006, 2008), taking the dilution factor and the absolute volume of DNA extract into account. The absolute 16S copy numbers were logcontrast-transformed (Aitchison 1986) and then subjected to discriminant analysis (SPSS) to test for quantitative differences in the microbial community composition across different populations or diets.

Table 1: Primers and probes used for the characterization (PCR, cloning, sequencing), quantification (qPCR), and localization (FISH) of bacterial taxa in the mid-gut of *P. apterus*. All primers target the bacterial 16S rRNA gene. Use: (1) general amplification of gut bacteria, (2) cloning/sequencing, (3) 454 sequencing, (4) qPCR, and (5) FISH.

Primer	Primer sequence (5´ - 3´)	Fwd/ Rev	5´mod	Target	Use	Reference
FD1	AGAGTTTGATCCTGGCTCAG	Fwd.		Eubacteria	1	Weisburg et al. 1991
RP2	ACGGCTACCTTGTTACGACTT	Rev.		Eubacteria	1	Weisburg et al. 1991
M13F	CAGGAAACAGCTATGAC	Fwd.		Eubacteria	2	Boutin-Ganache et al. 2001
M13R	GTAAAACGACGGCCAG	Rev.		Eubacteria	2	Boutin-Ganache et al. 2001
Gray28F	GAGTTTGATCNTGGCTCAG	Fwd		Eubacteria	3	Ishak et al. 2011
Gray519R	GTNTTACNGCGGCKGCTG	Rev		Eubacteria	3	Ishak et al. 2011
Corio_DSM20642_91F	TGACCAACCTGCCCTGCGCT	Fwd.		*Coriobacterium*	4	This study
Corio_300rev	CCCGTAGGAGTCTGGGCCG	Rev.		*Coriobacterium*	4	This study
Egg_1079fwd	CACTGCTGCCTCCCGTAGGAGT	Fwd		*Gordonibacter*	4	This study
Egg_1253Rev	CATACCTCACCTGGGGTGTGTGG	Rev		*Gordonibacter*	4	This study
Clostridium_1050-fwd	CTCGTGTCGTGAGATGTTGG	Fwd		*Clostridium*	4	This study
Clostridium_1248-rev	GCTCCTTTGCTTCCCTTTGT	Rev		*Clostridium*	4	This study
Proteobac_16s_fwd	GTGGCAAACGGGTGAGTAAT	Fwd		unknown Rickettsiales	4	This study
Proteobac_16s_Rev	GAAGTCTGGGCCGTATCTCA	Rev		unknown Rickettsiales	4	This study
Lactococcus_975-fwd	CGCTCGGGACCTACGTATTA	Fwd		*Lactococcus*	4	This study
Lactococcus_1175-rev	GCAGCAGTAGGGAATCTTCG	Rev		*Lactococcus*	4	This study
Klebsiella_250-fwd	CAGCCACACTGGAACTGAGA	fwd.		*Klebsiella*	4	This study
Klebsiella_453-rev	GTTAGCCGGTGCTTCTTCTG	Rev.		*Klebsiella*	4	This study
EUB338-Cy5	GCTGCCTCCCGTAGGAGT	Rev.	Cy5	*Eubacteria*	5	Amann et al. 1990
Cor653-Cy3	CCCTCCCMTACCGGACCC	Rev.	Cy3	*Coriobacterium*	5	Kaltenpoth et al. 2009
Egg583-Cy3	GAGGCTTCGCTTAGGCAACC	Rev.	Cy3	*Gordonibacter*	5	This study
Proteo-Cy3	ATTACTCACCCGTTTGCCAC	Rev.	Cy3	unknown Rickettsiales	5	This study
Clost -Cy3	TACCAACTCCCATGGTGTGA	Rev.	Cy3	*Clostridium*	5	This study
Lact_Cy3	GCTCCCTACATCTAGCAC	Rev.	Cy3	*Lactococcus*	5	Ercolini et al. 2003
Kleb_Cy3	TCTCAGTTCCAGTGTGGCTG	Rev.	Cy3	*Klebsiella*	5	This study

2.3.7 Fluorescence in-situ hybridization (FISH)

To localize the dominant symbionts of *P. apterus*, FISH was performed using sections of the M3 portion of the mid-gut. The M3 was fixed in 70% ethanol, dehydrated in acetone, and then embedded in cold polymerizing resin (Technovit 8100, Germany) according to

the manufacturer's instructions. Sections of 4 mm thickness were prepared with a diamond knife on a Microm HM 355 S microtome (Thermo Scientific, Germany). FISH on the embedded M3 mid-gut tissue sections was carried out using the specific probes *Cor*653, *Egg*583, *Proteo*, *Clost, Lact*, *Kleb* (all Cy3 probes), in combination with the general eubacterial probe EUB338-Cy5 (Amann *et al.* 1990) and DAPI (Table 1). Some of the probes were the same as one of the primers used for diagnostic qPCR (unknown *Rickettsiales* sp. and *Klebsiella sp.*); the others were designed based on the sequencing data available from the 454 pyrosequencing and cloning/sequencing (*Gordonibacter* sp. and *Clostridium sp.*). Sequences of probes for *Coriobacterium* sp. and *Lactococcus lactis* were derived from the literature (Ercolini *et al.* 2003; Kaltenpoth *et al.* 2009). The specificity of all probes was tested by using cultures of other bacterial taxa (*Escherichia coli*, *Bacillus subtilis* and *Pseudomonas fluorescens)* on eight-field microscope slides as negative controls, and a mid-gut (M3) suspension from an adult *P. apterus* as a positive control. Hybridization with probes was achieved as described (Kaltenpoth *et al.* 2009, 2012), and localization of bacterial taxa was recorded using an Axioimager Z1 fluorescence microscope (Carl Zeiss, Germany).

2.3.8 Microelectrode measurements

Freshly extracted guts of adult *P. apterus* individuals were placed on top of a 2 mm layer of 1.5% agarose in a micro-chamber and covered with 2 mm of 0.5% agarose (Brune *et al.* 1995). The microelectrode was positioned using a manual micromanipulator (Unisense, Denmark). The current was measured with a picoammeter (model 1201; Diamond-General, Ann Arbor, USA) connected to a strip chart recorder. An oxygen microelectrode (Unisense, Denmark) with a tip diameter of 20-30 µm was calibrated

before each experiment by using water saturated with air (21% O_2) or 100% N_2 (0% O_2), respectively. This set-up was used to radially measure oxygen concentrations of six replicates in the M3 region of the mid-gut using a step increment of 50 µm. A pH microelectrode (Unisense, Denmark) with a tip diameter of 20-30 µm was calibrated with standard buffers at pH 4.0, 7.0 and 10.0 before each experiment. The pH was recorded in three replicates in the mid-section of different gut regions (M1, M2, M3, and M4). All measurements were performed at ambient temperature (22 ± 1˚C).

2.4 RESULTS

2.4.1 Bacterial communities in different gut regions of *P. apterus*

The microbiota of different mid-gut regions (M1, M2, M3, and M4) of *P. apterus* were characterised using 454 pyrosequencing (Fig. 1). After quality trimming, a total of 52,357 bacterial 16S rRNA sequences were obtained, which were binned into a total of 78 OTUs after removing singletons and OTUs below 0.5% abundance (Table S1). The most dominant bacterial taxa present in the M1 and M2 were Alphaproteobacteria (*Brevundimonas sp.*, *Rhizobium sp.*, *Caulobacter sp.,* and *Ensifer sp.*), and Gammaproteobacteria (*Pseudomonas sp.* and *Hafnia sp.*). The M3 portion exhibited a unique bacterial community, which was dominated by Actinobacteria *(Coriobacterium glomerans* and *Gordonibacter sp.*), Alphaproteobacteria (Rickettsiales sp), and Firmicutes (*Clostridium sp.*) (Fig. 1 and 2). The microbiota of M4 was highly diverse and consisted of all major taxa found in the previous gut sections, i.e. Alphaproteobacteria (*Brevundimonas sp.* and *Rhizobium sp.*), Gammaproteobacteria (*Pseudomonas sp.*), Firmicutes (*Clostridium sp.* and *Lactococcus lactis*) and Actinobacteria (*Coriobacterium glomerans* and *Gordonibacter sp.*). Although female *P. apterus* have gastric caeca associated with the M4 region (Buchner 1965), no symbiotic bacteria could be detected in these structures by direct microscopic observations or FISH using general eubacterial probes (data not shown).

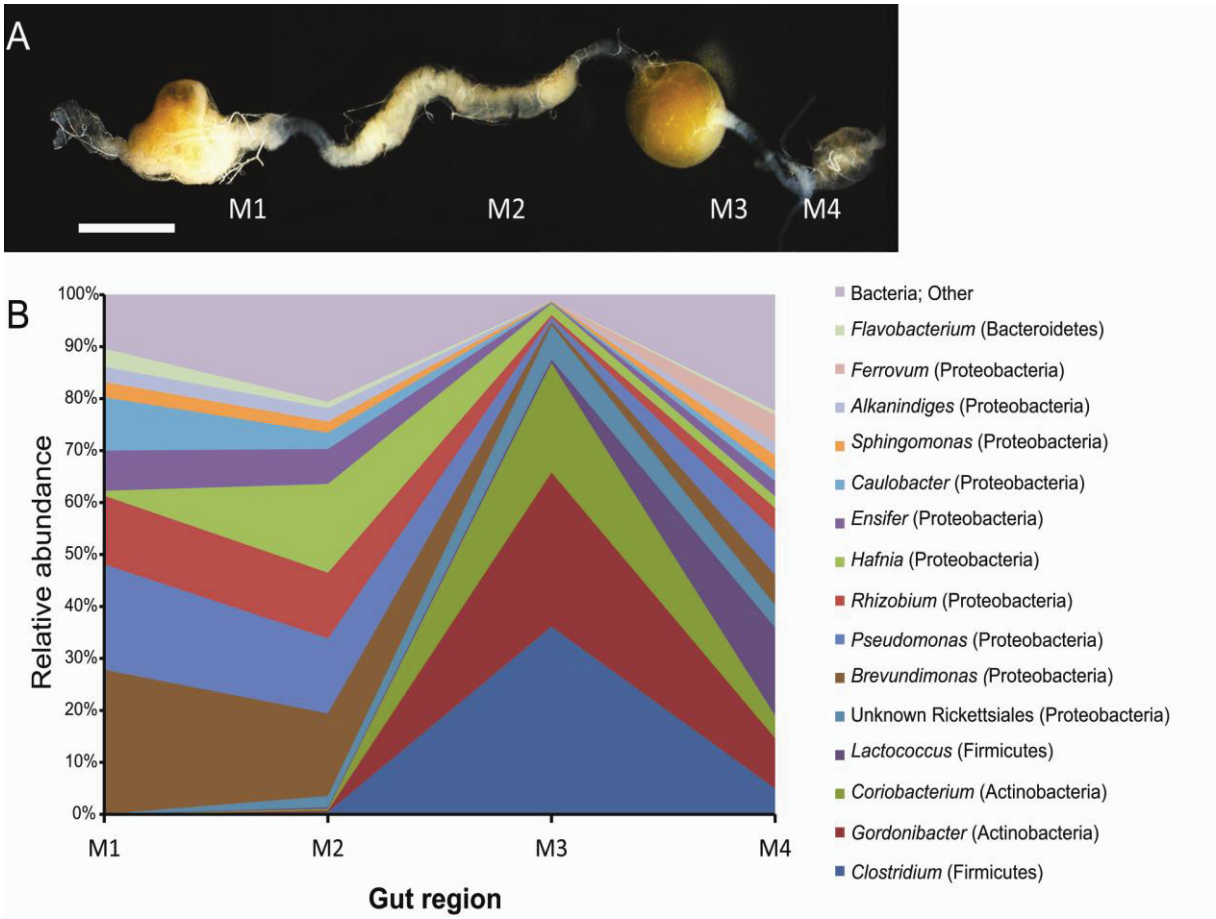

Figure 1. Bacterial community in different mid-gut regions of *P. apterus*. (**A**) Overview over the different parts of the digestive tract of *P. apterus* (M1-M4) (scale: 2mm). (**B**) Frequency of bacterial taxa in the four different regions of the mid-gut represented in a relative area graph as revealed by bTEFAP (52,357 16S rRNA reads in total) of a pooled sample of six adult individuals from Jena, Germany.

The major taxa in the M3 region were each represented by several closely related OTUs of different abundances (Table S2). Based on the available data, we cannot tell whether this observed microdiversity reflects true biological diversity or was caused by sequencing artefacts. However, for all of the individual analyses (gut regions, developmental stages, different populations and diets, respectively), the variation in OTU abundances was consistent across samples, with the same major OTU(s) representing the dominant bacterial genera. Thus, we decided to limit the further description of the results

as well as the discussion to genus-level patterns, in order to facilitate an understanding of our general findings. It should be noted, however, that our conclusions based on genus-level classifications are identical with those based on OTU level analyses, and all OTU tables (including all OTUs with abundances >0.5% in at least one of the samples) are available as supplementary tables (see Tables S1-S4).

The microbiota profile of complete adult bugs (both males and females) was very similar to the one of the isolated M3 mid-gut region (Fig. 3), indicating that the bacterial community present in the M3 by far outnumbers all other microbes present in *P. apterus*. Hence, we focused on the M3 region of the mid-gut to analyse in more detail whether the bug-associated microbiota changes across different life stages, populations, and diets.

The composition of the M3 microbiota obtained from the cloning/sequencing analysis was qualitatively similar to the 454 pyrosequencing data with the dominant phylotypes (*Coriobacterium glomerans*, *Gordonibacter sp.* and *Clostridium sp.*) all being detected, but there were considerable differences between both datasets in relative abundances of microbial taxa (Fig. S1).

2.4.2 Phylogenetic placement of *P. apterus* midgut symbionts

Based on near full-length 16S rRNA sequences obtained from the cloning/sequencing approach, a maximum likelihood tree including representative sequences of in- and outgroup taxa was reconstructed (Fig. 2). The *Coriobacterium* sequence was closely related to the type strain of *C. glomerans* that had been previously isolated from the intestinal tract of *P. apterus* by Haas and König (1987) and remains to date the only validly described species in the genus. The other actinobacterial taxon identified in the firebug gut (i.e. *Gordonibacter sp.)* shows only about 92% similarity to the 16S rRNA

gene sequence of its closest relative *Gordonibacter pamelaeae* (Würdemann *et al.* 2009), and 90-91% similarity to species in the genus *Eggerthella*. Both *Coriobacterium glomerans* and *Gordonibacter sp*. (Fig. 2) belong to the family Coriobacteriaceae (Actinobacteria), a group of anaerobic bacteria that is known to occur in the human intestine (Holdeman *et al.* 1976) and as opportunistic pathogens in human oral infections (Downes *et al.* 2001; Nakazawa *et al.* 1999; Poco *et al.* 1996). But to our knowledge these bacterial taxa have not yet been reported from insects outside the Pyrrhocoridae family.

Firmicutes that were detected in the firebug´s gut as well as the gamma-proteobacterial symbionts are closely related to cultured strains (96% similarity to *Clostridium hathewayi*, 100% to *Lactococcus lactis*, and 100% to *Klebsiella pneumoniae*, respectively, Fig. 2). However, the alpha-proteobacterial taxon is only distantly related to any other known strain within the order Rickettsiales, with the closest relatives being an uncharacterized symbiont of the flea *Oropsylla hirsuta* (90% similarity, Jones *et al.* 2008), and *Holospora obtusa*, an intracellular symbiont of *Paramecium* (79% similarity, Amann *et al*. 1991). In general, the order Rickettsiales comprises mostly intracellular bacteria such as *Wolbachia* and *Rickettsia,* which are common reproductive parasites and pathogens of insects as well as other animals including humans (Hilgenboecker *et al.* 2008; Hosokawa *et al.* 2010b; Rousset *et al.* 1992). However, mutualistic interactions with *Wolbachia* and Rickettsia are known to occur in nematodes, leeches and bed bugs (Hosokawa *et al.* 2010b; Kikuchi *et al*. 2002; Taylor *et al.* 2005).

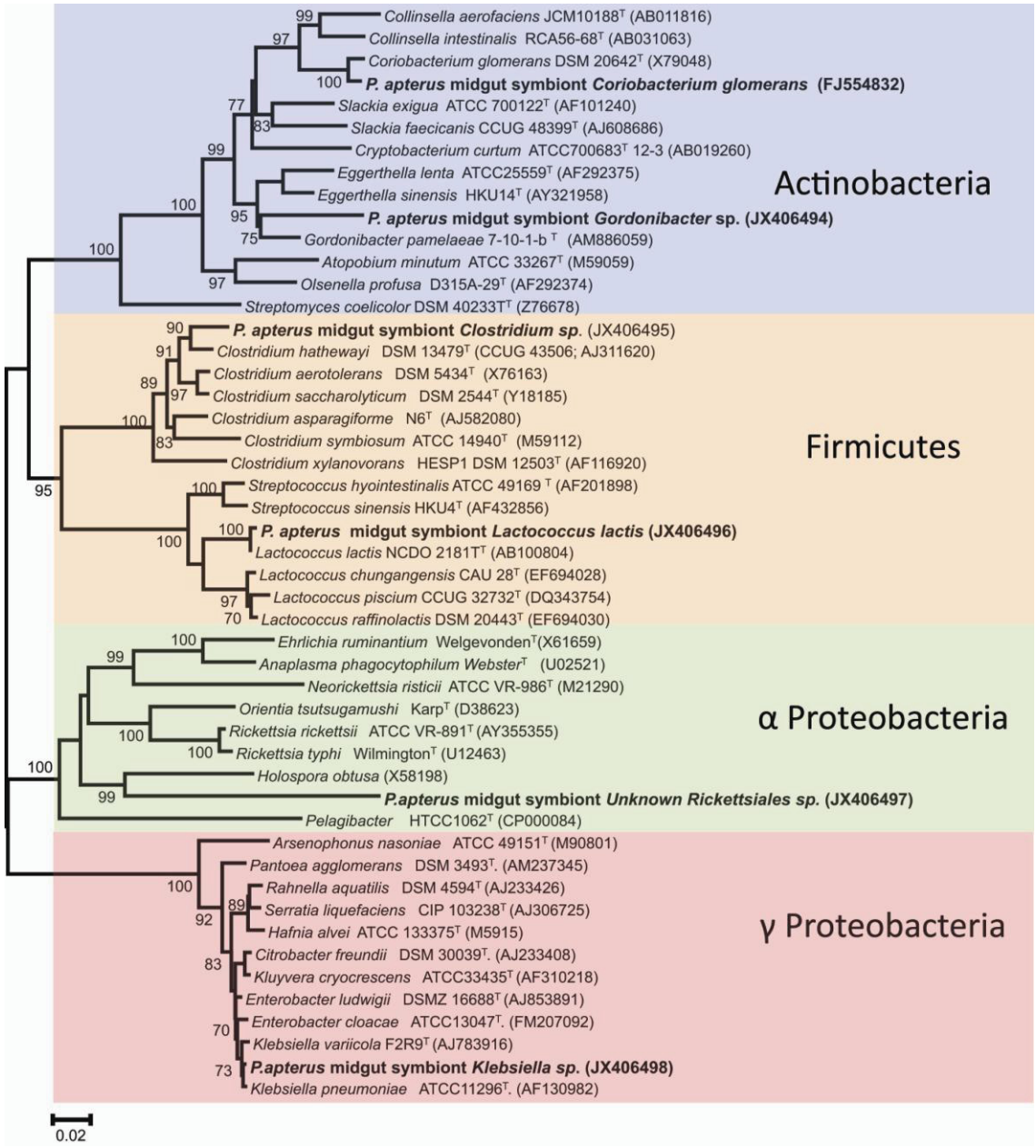

Figure 2. Phylogenetic position of symbiotic *Coriobacterium glomerans, Gordonibacter sp, Unknown Rickettsiales, Clostridium sp., Lactococcus sp,* and *Klebsiella sp.* from the *Pyrrhocoris apterus* mid-gut M3 region. Maximum likelihood tree constructed on the basis of 1.4 kbp of 16S rRNA gene sequences. Bootstrap values (in percent) were obtained from a search with 500 replicates. Strain and accession numbers are given behind the species names. Type strains are indicated by superscript T.

2.4.3 Ontogenetic changes of the *P. apterus* microbiota

Using qPCR and 16S rRNA data (90,899 16S rRNA sequences obtained by 454 pyrosequencing, clustered into 176 OTUs), we analysed the relative abundances of bacterial taxa across the different developmental stages of *P. apterus* in order to assess the symbionts' population dynamics within the insect host. The most abundant taxa *Clostridium sp.*, *Coriobacterium glomerans, Gordonibacter sp.,* as well as the next three most abundant bacterial taxa i.e. the unknown Rickettsiales species, *Lactococcus lactis,* and *Klebsiell*a *sp.* were detected in or on *P. apterus* eggs, suggesting that they are vertically transmitted from mother to offspring (Fig. 3, Table S2). Although the gut microbiota was present in low abundances from the egg stage to the second instar, the microbial community of *P. apterus* that characterizes adult individuals was quantitatively established during the 2nd or 3rd instar and remained largely unchanged until *P. apterus* reached its adult stage (Fig. 3).

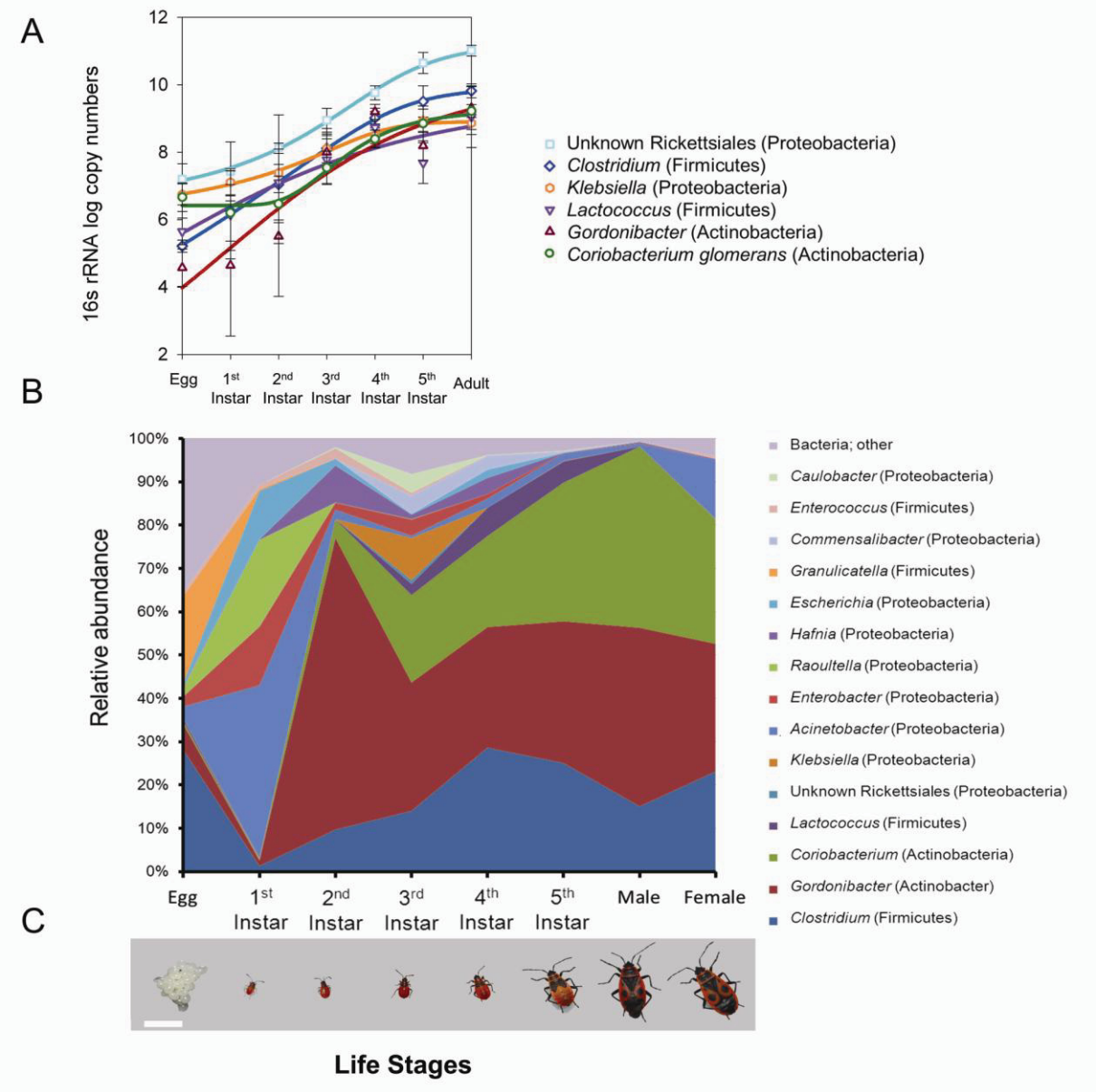

Figure 3. Bacterial community composition of *P. apterus* during its different life stages. (**A**) Ontogenetic change of the six major bacterial taxa across different life stages of *P. apterus* as revealed by qPCR using six replicates per life stage, with sigmoidal curve fitting (five parameters). (**B**) Relative abundance of bacterial taxa across different life stages of *P. apterus* (90,899 sequences in total) represented in a relative area graph of a pooled sample of six replicates per life stage. (**C**) Images of different life stages of *P. apterus*. Scale: 5mm.

2.4.4 Inter-population differences in the mid-gut microbiota of *P. apterus*

After quality trimming, a total of 83,174 bacterial 16S rRNA sequences and 103 OTUs were obtained from pooled samples of five different populations in Central Europe (Table S3). A quantitative and qualitative comparison of the gut microbiota revealed only minor differences across populations. The most abundant taxa *Clostridium sp.*, *Coriobacterium glomerans, Gordonibacter sp.,* and the next three most abundant bacterial taxa (i.e. unknown Rickettsiales sp., *Lactococcus lactis,* and *Klebsiell*a *sp.*) were consistently present across all populations (Fig. 4A,B). QPCR analysis showed that all six bacterial taxa were present in similar abundances across all populations (Fig. 4B). Discriminant analysis of the 16S log copy numbers obtained from qPCRs showed slight, but significant quantitative differences in the microbial communities derived from Central European bug populations (Wilk‘s λ =0.337, χ^2 = 36.5, n = 45, P = 0.049) (Fig. 4C).

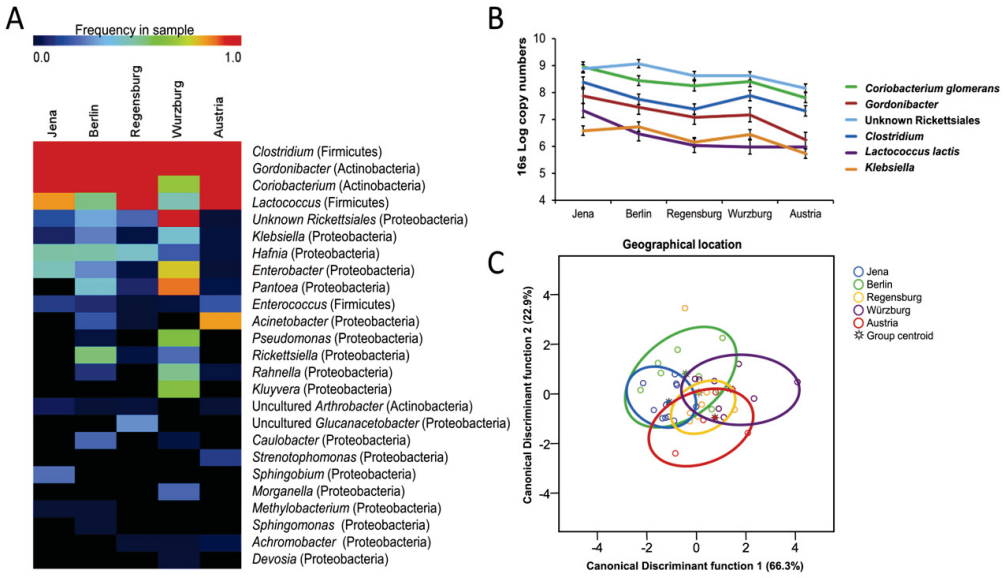

Figure 4. Gut bacterial community composition of *P. apterus* collected from five different geographical locations. (**A**) Relative abundance of gut bacterial taxa from 454 pyrosequencing of 16S rRNA amplicons (83,174 reads in total), represented as a heat map based on the log-transformed values, with warm colors indicating higher and cold colors lower abundance. (**B**) Infection rate and abundance of the six most dominant bacterial taxa (*Coriobacterium glomerans, Gordonibacter sp.,* unknown Rickettsiales, *Clostridium sp., Lactococcus sp.* and *Klebsiella sp.*) as revealed by diagnostic qPCR of multiple specimens for each population of *P. apterus* (Jena (n=6), Berlin (n=8), Regensburg (n=8), Würzburg (n=8) (all in Germany) and Maria Saal (n=5) (Austria)). Lines represent means, error bars denote standard errors. (**C**) Multivariate discriminant analysis of the microbial community of *P. apterus* from five geographic localities based on the qPCR data from (B) (Wilk's λ =0.337, χ^2 = 36.5, n = 45, *P* = 0.049).

2.4.5 Effect of different diets on the mid-gut microbiota of *P. apterus*

The mid-gut microbiota of experimental *P. apterus* populations fed exclusively on one of three different diets (linden seeds, sunflower seeds, and beewolf larvae, respectively) were analysed by 454 pyrosequencing and qPCR. The 454 pyrosequencing data (46,423 bacterial 16S rRNA sequences and 75 OTUs after quality trimming) revealed that the microbiota of *P. apterus* reared on the three different diets were similar, with *Clostridium* sp., *Coriobacterium glomerans* and *Gordonibacter sp.* being abundant in all three populations (Fig. 5A, Table S4). However, bugs fed on linden seeds and beewolves, respectively, showed unusually high abundances of gamma-Proteobacteria in the M3 region. This was especially surprising in the linden seed treatment, as Proteobacteria were much less common in the other experimental bugs from the same population and food source (see sections on the microbial community of different gut regions and populations).

Although the Rickettsiales *sp.*, *Klebsiella sp.* and *Lactococcus lactis* were not detected in the 454 pyrosequencing dataset, they were consistently found by qPCR of the same samples. The results of the qPCR analysis indicated that the six most dominant bacterial taxa were present in similar abundances across the different diet treatments, thereby indicating that the community composition of these taxa was not affected by the diet of the host (Fig. 5B). Discriminant analysis confirmed that there was no significant difference in the relative abundances of the six major bacterial taxa across diets (Wilk‘s $\lambda = 0.543$, $\chi^2 = 7.029$, $n = 45$, $P = 0.134$) (Fig. 5C).

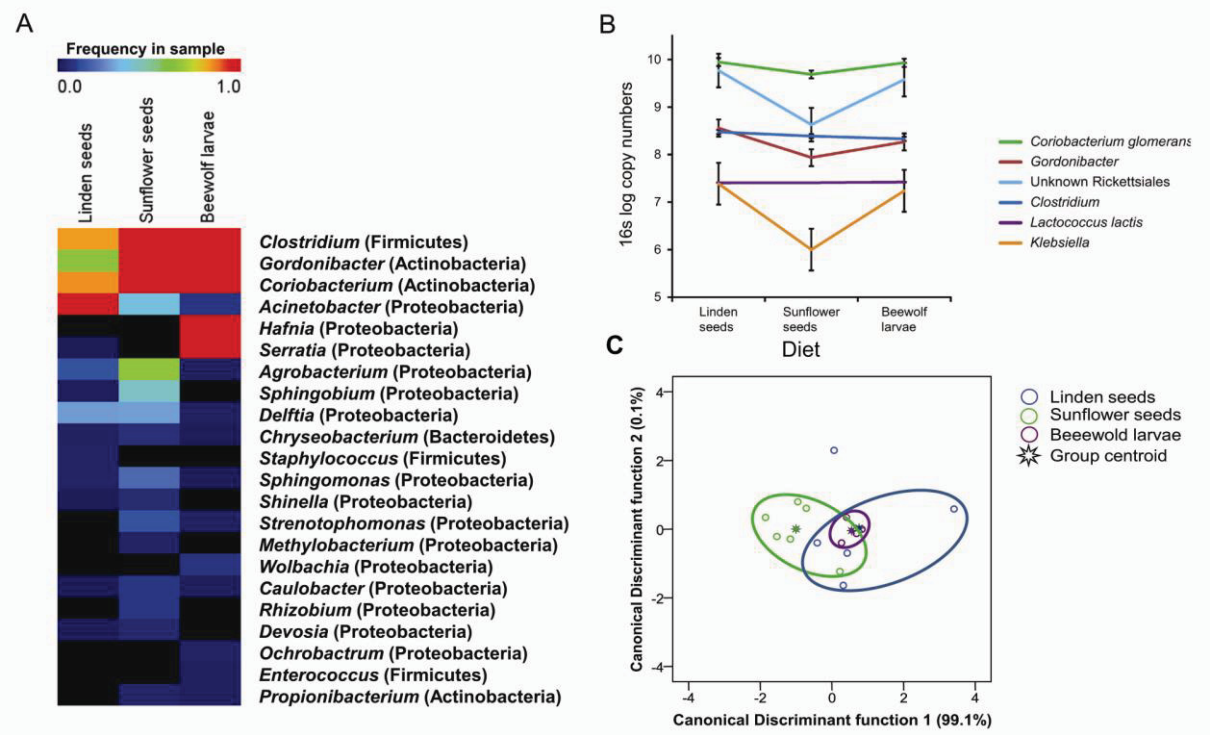

Figure 5. Gut bacterial community composition of *P. apterus* reared on three different diets. (**A**) Frequency of gut bacterial taxa from 454 pyrosequencing data (46,423 sequences in total) represented as a heat map based on the log-transformed values, with warm colors indicating higher and cold colors lower abundance. (**B**) Infection rate and abundance of the six most dominant bacterial taxa (*Coriobacterium glomerans, Gordonibacter sp.,* Unknown Rickettsiales, *Clostridium sp., Lactococcus* sp., and *Klebsiella sp.*) as revealed by diagnostic qPCR of five replicates for each different diet. Lines represent means, error bars denote standard errors. (**C**) Multivariate discriminant analysis of the microbial community of *P. apterus* reared on three different diets based on the qPCR data from (B) (Wilk's $\lambda = 0.543$, $X^2_{45} = 7.029$, $P = 0.134$, 66.7% of cases classified correctly).

2.4.6 Transient microbes from ingested food material

The microbiota of the preferred food source of *P. apterus* (linden seeds) was analyzed by 454 pyrosequencing (Table S5) to assess the possible influence of transient microbes from the ingested food material in the symbiotic gut microbial community. After quality trimming, a total of 12,017 16S rRNA sequences were obtained. Around 47% of the sequences originated from chloroplasts; these sequences were excluded from further

analysis. Some of the most abundant bacterial taxa identified were Gammaproteobacteria (*Pseudomonas sp.*, *Stenotrophomonas sp.*), Alphaproeobacteria (*Sphingomonas sp.*, *Methylobacterium sp.*), and Actinobacteria (*Microbacterium sp.*, *Rhodococcus sp.*). Several bacterial taxa that could be detected in linden seed samples were also constituents of the microbiota of M1, M2, and M4 mid-gut regions, notably *Brevundimonas sp.*, *Rhizobium sp.*, *Pseudomonas sp.*, and *Caulobacter sp.* (Table S5).

Interestingly, *Wolbachia sp*. was one of the bacterial taxa identified in the *P. apterus* individuals reared on beewolf larvae as the sole food source (Fig. 5A). As beewolves are commonly infected with *Wolbachia* (Kaltenpoth 2006), it is likely that the sequences found in *P. apterus* represent transient bacteria or DNA residues acquired from the food source. Both the occurrence of linden seed-associated bacteria in the mid-gut of bugs feeding on these seeds and the discovery of *Wolbachia* in beewolf-fed bugs highlight the possible confounding effect of transient microbes on the apparent composition of gut microbial communities. This effect should be especially problematic in studies analyzing microbial gut communities based on low numbers of field-collected samples that were exposed to diverse and unknown microbial communities.

2.4.7 Localisation of microbial symbionts

The numerically most dominant bacterial taxa found in the M3 mid-gut portion of *P. apterus* (*Clostridium sp.*, *Gordonibacter sp.*, *Coriobacterium glomerans*, unknown Rickettsiales *sp.*, *Lactococcus lactis* and *Klebsiella sp.)* were localized in gut sections using FISH. *C. glomerans* and *Gordonibacter* sp. were present in the gut lumen and on the epithelial walls, with *C. glomerans* being predominantly localized towards the anterior end of the M3. The unknown Rickettsiales *sp.*, *Klebsiella sp.*, *Lactococcus lactis*

and *Clostridium* sp. were present in the gut lumen, with *Lactococcus lactis* and *Clostridium* sp. being particularly prevalent in the anterior region of the M3 (Fig. S2).

2.4.8 Microelectrode measurements

Radial oxygen measurements in the M3 region of the *P. apterus* mid-gut indicated that the conditions were completely anoxic throughout the M3 (Fig. S3). The pH measurement in the mid-gut of *P. apterus* revealed slightly acidic conditions throughout the mid-gut. The M3 region of the mid-gut had a pH of 5.8, while the other regions of the mid-gut (M1, M2, and M4) showed a pH of 5.4. The acidic pH conditions in the mid-gut region are required for the effective functioning of the digestive enzymes (Kodrík *et al.* 2012; Silva and Terra 1994).

2.5 DISCUSSION

2.5.1 Composition of the *P. apterus* gut microbiota

In this study, we comprehensively characterized the microbiota inhabiting the mid-gut of *P. apterus* using a combination of different culture-independent techniques. The M1 and M2 mid-gut regions showed similar microbial profiles that were dominated by Alpha- and Gammaproteobacteria (Fig. 1). Many of the bacterial taxa were also found in the microbiota of the bug's food source (linden seeds), suggesting that the bacterial taxa detected in the M1 and M2 region were taken up with the food. The microbiota of the M4 mid-gut region shared bacterial taxa with all preceding mid-gut regions (Fig. 1). The presence of such a diverse bacterial community in M4 is likely due to the passing of bacteria from the other gut regions with the digested food material to the rectum for excretion. However, the M3 region was characterized by a distinct microbiota compared to the microbiota present in the other mid-gut regions and the food source, and predominantly harboured *Coriobacterium glomerans*, *Gordonibacter sp., Clostridium sp.,* as well as *Lactococcus lactis,* an unknown Rickettsiales species, and *Klebsiella sp.* (Fig. 1 and 2). Furthermore, the microbial profile of the M3 mid-gut region resembled the microbiota of the whole adult insect (male and female) (Fig. 3), indicating that the symbionts residing in the M3 greatly outnumber any other bacterial taxa occurring in other parts of the bug. Interestingly, *Dysdercus fasciatus*, another species within the Pyrrhocoridae, harbours a mid-gut M3 microbiota similar to *P. apterus*, with the most abundant bacterial taxa being *Coriobacterium glomerans*, *Gordonibacter sp*., and *Clostridium sp*. (Kaltenpoth et al. 2009; Salem *et al.* submitted).

By contrast, other pentatomomorphan bugs are associated with proteobacterial symbionts that inhabit specialized structures such as gastric caeca or crypt regions in the posterior

region of the midgut (Buchner 1965; Glasgow 1914; Kikuchi *et al.* 2011a). As in pyrrhocorid bugs (Salem et al., submitted), the symbionts are often essential for successful growth and reproduction of the host (Abe *et al.* 1995; Fukatsu and Hosokawa 2002; Huber-Schneider 1957; Kikuchi *et al.* 2009; Muller 1956; Schorr 1957; Tada *et al.* 2011). In the superfamily Pentatomoidea, most of the species are associated with one of several distinct lineages of γ-Proteobacteria that are vertically transmitted (Fukatsu and Hosokawa 2002; Kikuchi *et al.* 2009; Prado *et al.* 2006; Prado and Almeida 2009). Bugs of the superfamily Coreoidea and several families of the Lygaeoidea, on the other hand, harbour *Burkolderia* (β-Proteobacteria) symbionts in the mid-gut crypts, and it has been shown for some broad-headed bugs (Alydidae) that these symbionts are acquired *de novo* from the environment in every host generation (Kikuchi *et al.* 2011a). However, other families of Lygaeoidea have secondarily lost the crypt-inhabiting symbionts and evolved bacteriomes housing a distinct clade of γ-Proteobacteria (Kuechler *et al*. 2012; Matsuura *et al*. 2012).

In contrast, no such bacteriomes could be detected in *P. apterus* and the gastric caeca constitute small and little developed invaginations that are present only in females and appear to be devoid of any bacteria (Buchner 1965, this study). Hence, our results show that the symbiotic microbiota of Pyrrhocoridae bugs namely *P. apterus* and *D, fasciatus* is markedly different from bugs in other superfamilies within the infraorder Pentamomorpha (Fukatsu and Hosokawa 2002; Hosokawa *et al.* 2010a; Kikuchi *et al.* 2011a; Prado and Almeida 2009), with regard to both the localization of the symbionts in the M3 region of the mid-gut and the composition, consisting predominantly of Actinobacteria and Firmicutes as well as γ- and α-Proteobacteria. This indicates that the microbiota represented in the mid-gut M3 region of *P. apterus* could be specific to bugs in the

superfamily Pyrrhocoroidea, and the relationship between gut microbes and pyrrhocorid bugs may represent an ancient and possibly coevolved symbiotic community.

2.5.2 Transmission and establishment of the microbial mid-gut community in *P. apterus*

In order to gain a better understanding of the transmission route and establishment of the symbiotic community within *P. apterus*, we characterized the microbiota in different life stages of the bug. In many species of stinkbugs, the hosts are highly dependent on their microbial partners, and experimental removal of the symbionts has been shown to result in retarded growth and/or high mortality (Abe *et al.* 1995; Fukatsu and Hosokawa 2002; Huber-Schneider 1957; Kikuchi *et al.* 2009; Muller 1956; Schorr 1957; Tada *et al.* 2011). To ensure infection of their offspring with the symbiotic microbes, most of these bugs evolved a vertical transmission route via egg smearing, coprophagy, or the deposition of symbiont-containing capsules (Abe *et al.* 1995; Hosokawa *et al.* 2005; Kikuchi *et al.* 2009; Prado *et al.* 2006; Schorr 1957). In the case of *P.apterus*, the presence of the six most dominant gut bacterial taxa already within the eggs or on the surface indicates that they are likely transmitted vertically from mother to offspring, which has been shown previously for *C. glomerans* (Kaltenpoth *et al.* 2009).

A high diversity of bacterial taxa was detected in the nymphs until the second instar, before the symbiotic bacterial community became well-defined between the second and the third larval instar stage. This is in accordance with direct observations of the feeding behaviour in *P. apterus* nymphs, as the nymphs start feeding on linden seeds only in the 2nd instar stage (Puchkov 1974). Similarly successful establishment of the symbiont in the second instar has been observed in the bean bug (*Riptortus pedestris*) (Kikuchi *et al.*

2011b). Even though in this case the symbiont is acquired horizontally from the environment, acquisition and establishment of the symbionts in later instars were much less effective and could lead to fitness costs for the host (Kikuchi *et al.* 2007; Kikuchi *et al.* 2011b). As newborn nymphs possess a high amount of yolk in their gut and can develop into the second instar without feeding, nutritional symbionts are probably not essential during this developmental period (Kikuchi *et al.* 2011b; Leal *et al.* 1995). Although the symbionts are already present in first instar *P. apterus*, they do not increase to significant abundances until the larva have reached second/third instar. Thus, the microbial community only becomes well-defined upon the initiation of feeding on the herbivorous diet, when the symbionts probably begin to play an important role in supplementing limiting nutrients to the bug (Salem et al., submitted).

2.5.3 Ecological stability of the *P. apterus* gut microbiota

The insect gut is constantly exposed to different diets and to transient microbes that could significantly affect the composition of the indigenous microbiota. This has been evident in several systems such as in mosquitos (*Aedes albopictus* and *A. aegypticus*, Zouache *et al.* 2011) and chestnut weevils (*Curculio sikkimensis*) (Toju and Fukatsu 2011), where the microbial communities vary with the geographical location and other ecological parameters. Similarly, in European corn borer moths the bacterial community structure differed between lab-reared and wild type hosts (Belda *et al.* 2011). In cockroaches, the supply with an artificial diet that was low in protein and high in fibre content resulted in predictable alterations in the microbial gut community and concomitant changes in gut physiology, with a decline of streptococcal and lactobacillus symbionts from the foregut resulting in a decrease in the production of lactate and acetate (Kane and Breznak 1991). Similarly, the hindgut microbiota of crickets changed significantly with the diet, which

resulted in a reduction of hydrogen and carbon dioxide production (Santo Domingo *et al.* 1998).

Our results indicate that the gut microbiota of *P. apterus* is both qualitatively and quantitatively remarkably stable across different populations and diets. Although there were some diet-associated changes in the bacterial community, the six dominant microbial strains were consistently abundant. This ecological stability of the microbial community implies functional importance of the dominant taxa, which is supported at least for the actinobacterial symbionts by experimental manipulation of the microbiota and subsequent fitness assays (Salem et al. submitted). Over evolutionary timescales, functionally relevant and vertically transmitted microbial symbionts are expected to co-evolve with their host, resulting in a congruence of microbial communities on higher taxonomic levels. Such patterns with shared microbial core communities across different species have recently been suggested for ants (Anderson *et al.* 2012), honey bees and bumble bees (Martinson *et al.* 2011) as well as termites and some closely related cockroaches (Schauer *et al.* 2012). Future studies on other Pyrrhocoridae species will allow for an identification of long-term associated microbial symbionts with this family of bugs and reveal the evolutionary history of this association.

2.5.4 Comparison of methods for the characterization of microbial communities

Comparative analyses of the 454 pyrosequencing and the qPCR data of different populations, diets, and life stages of *P. apterus* showed differences in the relative abundances of the dominant bacterial taxa in qPCR and 454 pyrosequencing, indicating that relative abundances obtained by 454 amplicon sequencing alone have to be considered with caution when analyzing the composition of unknown microbial

communities (Amend *et al.* 2010; Zhou *et al.* 2011). In addition, some of the dominant bacterial taxa that were consistently present in the M3 region of the mid-gut were not always detected by bTEFAP 454 pyrosequencing, although their presence in the gut could be demonstrated by qPCR. Furthermore, the analysis of the 454 pyrosequencing data revealed multiple OTUs representing each of the most dominant bacterial taxa. Even though this pattern was consistent across different samples and may therefore reflect true biological microdiversity within the gut of *P. apterus*, we cannot exclude the possibility of sequencing artefacts and noise causing an overestimation of bacterial OTUs (Gilles *et al.* 2011; Quince *et al.* 2011), so further analyses are necessary to establish strain-level associations and symbiont microdiversity within *P. apterus*. Thus, even though bTEFAP is an advanced high throughput technique that allows for rapid, cost-effective, and detailed analyses of complex microbial communities, it suffers from similar flaws as the traditional cloning and sequencing approach (PCR biases and other possible confounding factors, as well as a higher rate of sequencing errors), and the results therefore have to be considered with caution (Amend *et al.* 2010; Zhou *et al.* 2011). The combination of several molecular techniques (bTEFAP, cloning/sequencing, diagnostic PCR) is advisable for an accurate characterization of microbial communities.

2.5.5 Putative function of the *P. apterus* mid-gut microbiota

Symbiotic gut microbes can assist their host to subsist on suboptimal diets by increasing its digestion efficiency, detoxifying plant allelochemicals, or by providing digestive enzymes or limiting nutrients (Douglas 1992). Plant material is often low in nitrogen, essential amino acids, B vitamins and sterols (Douglas 1998). However, many herbivorous insects are associated with symbiotic microbes that possess the metabolic abilities to synthesize these compounds and thereby enable the insects to exploit

otherwise inaccessible food sources (Douglas 1992; Jones 1984). In several insect taxa, e.g. in crickets, termites and cockroaches, the gut microbiota are involved in breaking down the ingested polysaccharides, notably lignocellulose, the most abundant biological polymer on earth, and fermenting the resulting monosaccharide mixture into short chain fatty acids (Bäckhed *et al.* 2005; Brune and Friedrich 2000; Kaufman and Klug 1991; Nalepa *et al.* 2001). In this mutualistic relationship, the host gains carbon and energy, while the microbes are provided with a rich source of glycan, a protected anoxic environment, as well as a reliable transmission into the next generation of host insects.

Pyrrhocorid bugs preferentially feed on seeds of Malvales plants, which are generally avoided by other phytophagus insects due to the detrimental effects of their phytochemical defenses such as gossypol and cyclopropenoic fatty acids, which can interfere with the digestion of food materials, cause retarded growth, and lead to sterility (Abou-Donia 1976; Allen *et al.* 1967; Kristenová *et al*. 2011). Our comparative analysis of the different gut regions and the microbiota of the ingested food material suggest that specific symbiotic bacteria are restricted to the M3 region, where the food particles also remain for the longest period of time (Kodrík *et al.* 2012; Silva and Terra 1994). Previous studies have shown that the experimental removal of the microbiota by sterilizing the egg surface had a strong negative effect on the fitness of *P. apterus*, as the aposymbionts showed an increased mortality as well as delayed development in comparison to individuals with the native microbiota (Kaltenpoth et al. 2009, Salem *et al.*, submitted).

The mid-gut M3 of *P. apterus* is a completely anoxic environment ideal for fermentation (Fig. S3), and consequently, the resident bacterial taxa are either facultative or obligate anaerobes. Thus, the bacterial taxa residing in the M3 region could play an important role for the insect by degrading complex dietary components, providing nutrient supplementation, or detoxifying noxious chemicals (e.g. cyclopropenoic fatty acids or

gossypol) in the diet. Targeted manipulation experiments of the bacterial community in bugs fed on different food sources indicated that the differences in growth rate and survival between aposymbiotic and symbiotic individuals are likely due to a nutritional contribution of the actinobacterial symbionts rather than the detoxification of plant secondary metabolites (Salem et al., submitted). Thus, the symbionts may enable their insect hosts (Pyrrhocoridae) to exploit and diversify in a specific ecological niche (Malvales plants) that is inaccessible to many other insects.

As several members of the Pyrrhocoridae are pests of economically important crops such as cotton (*Gossypium hirsutum*) and okra (*Abelmoschus esculentus)*, elucidating the role of the gut microbiota of pyrrhocorid bugs could provide valuable information to be used in biocontrol. Furthermore, since the vast majority of eukaryote-bacteria symbioses likely constitute complex multipartite rather than one-host/one-symbiont interactions (Ferrari and Vavre 2011), pyrrhocorid bugs represent one of the few established and experimentally amenable systems so far that can be used to address fundamental questions on the functional roles and the interactions of multiple bacterial symbionts within an insect host.

2.6 ACKNOWLEDGEMENTS

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2.8 SUPPLEMENT

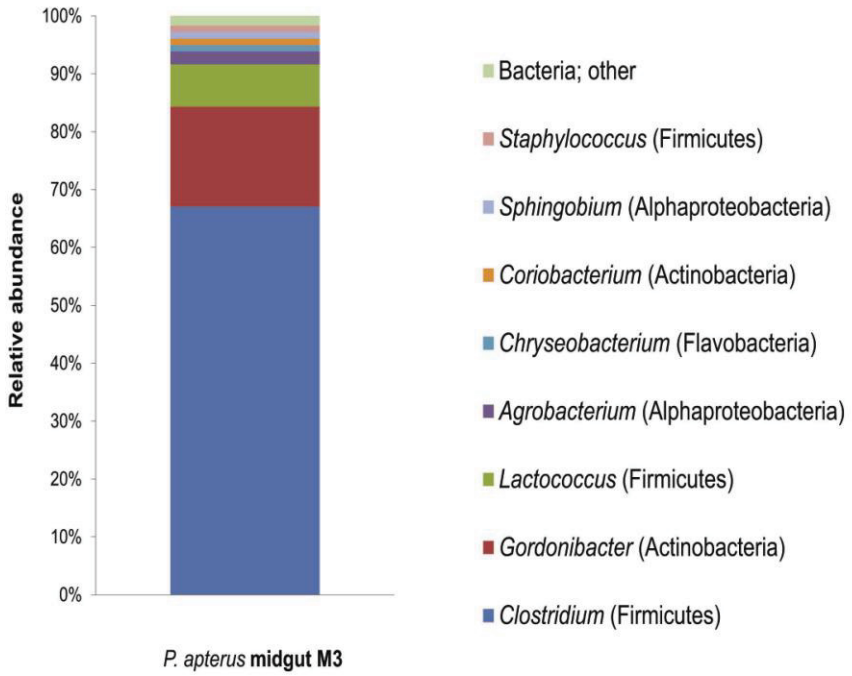

Fig. S1 Composition of the gut microbial community of *Pyrrhocoris apterus* as characterised by using PCR amplification with general eubacterial primers followed by cloning and capillary sequencing [based on 179 near full-length 16S rRNA gene sequences (1.4 kbp)].

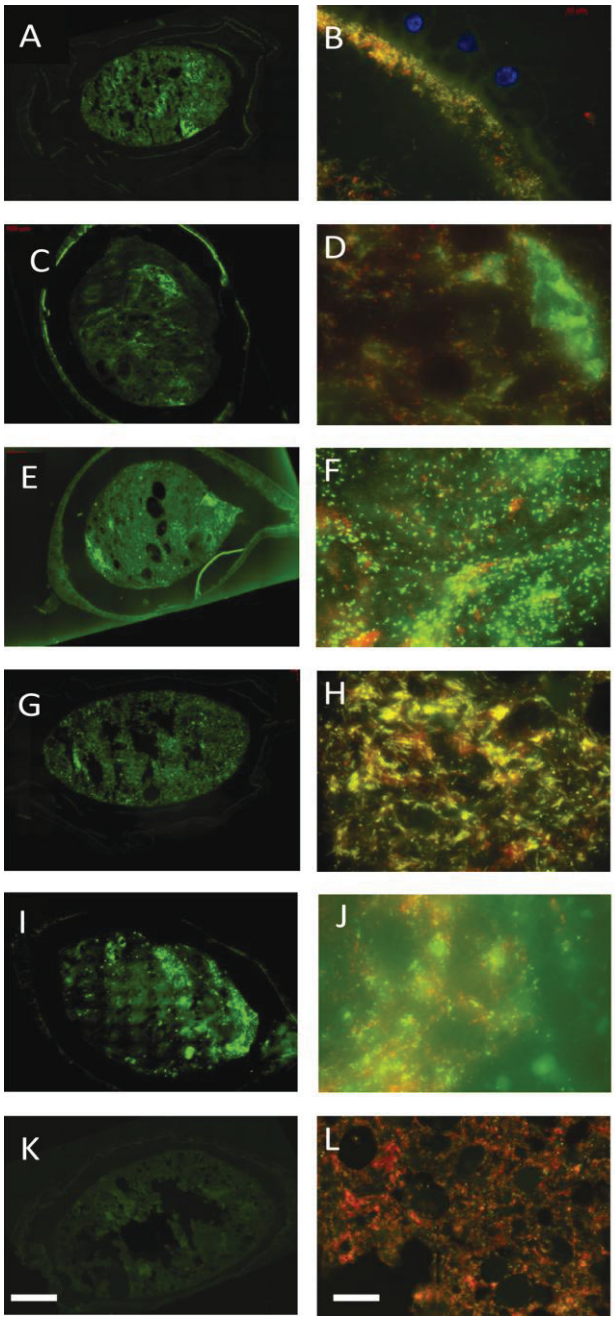

Fig. S2 Fluorescence *in situ* hybridization of complete mid-gut M3 sections (left panel) and close-up views of bacterial cells (right panel) after staining with specific probes (green) for *Coriobacterium glomerans* (A, B) *Gordonibacter* sp. (C, D), *Clostridium* sp. (E, F), Rickettsiales sp., (G, H), *Lactococcus lactis* (I, J), and *Klebsiella* sp. (K, L) as well as the general eubacterial probe EUB338 (red) and DAPI (blue). For the complete mid-gut pictures (left panel), only the green channel was included in the picture. Scale bars: left panel: 100 µm, right panel: 10 µm.

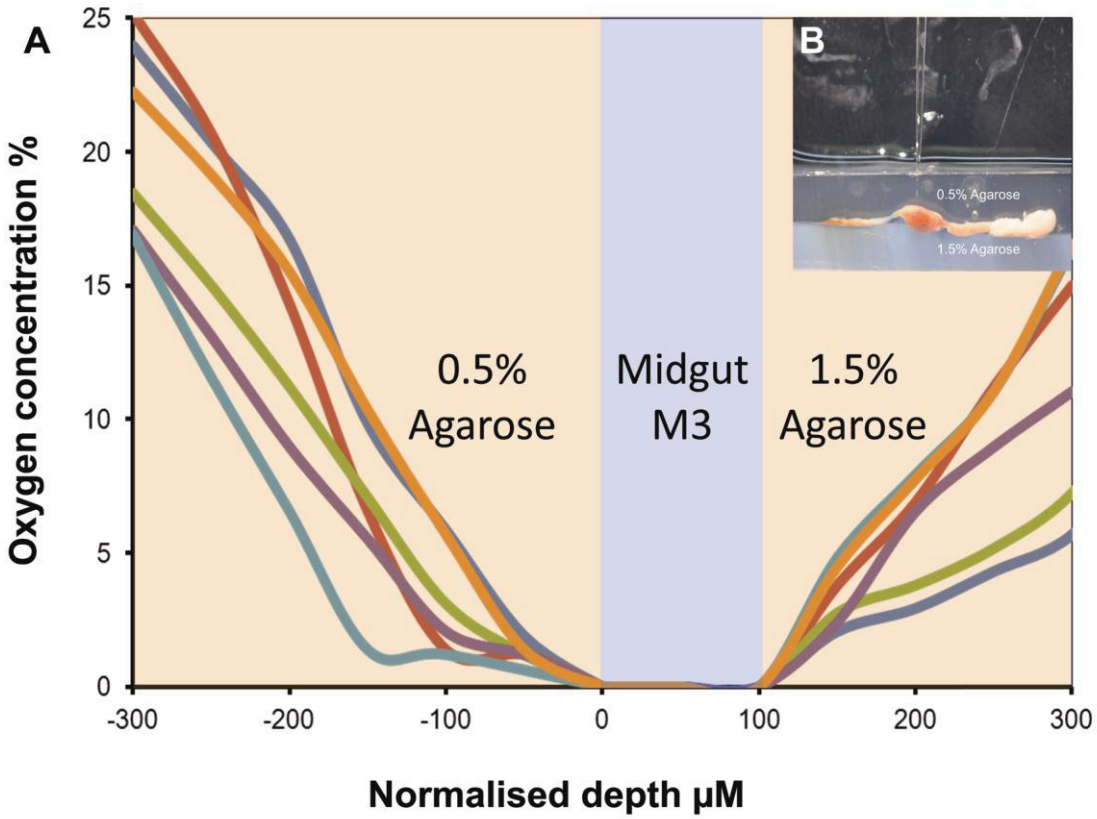

Fig. S3 Oxygen content in the mid-gut M3 region of *Pyrrhocoris apterus*. (A) Radial oxygen measurement in the mid-gut M3 region of six different *P. apterus* individuals. The step increments were 50 µm. (B) Image of the microelectrode set-up used for the oxygen measurement in the mid-gut M3 region.

CHAPTER 3

Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae)

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Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae)

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3.1 ABSTRACT

Actinobacteria engage in defensive symbioses with several insect taxa, but reports of nutritional contributions to their hosts have been exceptionally rare. Cotton stainers (*Dysdercus fasciatus*) and red firebugs (*Pyrrhocoris apterus*) (both Hemiptera, Pyrrhocoridae) harbor the actinobacterial symbionts *Coriobacterium glomerans* and *Gordonibacter* sp.as well as Firmicutes (*Clostridium* sp. and *Lactococcus* sp.) and Proteobacteria (*Klebsiella* sp. and a Rickettsiales bacterium) in the M3 region of their mid-gut. We combined experimental manipulation with community-level analyses to elucidate the function of the gut symbionts in both pyrrhocorid species. Elimination of symbionts by egg-surface sterilization resulted in significantly higher mortality and reduced growth rates, indicating that the microbial community plays an important role for host nutrition. Fitness of symbiont-deprived bugs could be completely restored by re-infection with the original microbiota, while reciprocal cross-infections of microbial

communities across both pyrrhocorid species only partially rescued fitness, demonstrating a high degree of host-symbiont specificity. Community-level analyses by quantitative PCRs targeting the dominant bacterial strains allowed us to link the observed fitness effects to the abundance of the two actinobacterial symbionts. The nutritional mutualism with Actinobacteria may have enabled pyrrhocorid bugs to exploit Malvales seeds as a food source and thereby possibly allowed them to occupy and diversify in this ecological niche.

3.2 INTRODUCTION

Insects, the most abundant animal class on earth, engage in a remarkable diversity of symbiotic associations involving microbial partners (Buchner, 1965). Many of these partnerships benefit the insect host by improvements to the metabolism, physiology and catabolic capacity through nutritional supplementation or the degradation of complex dietary compounds (Douglas, 1998; 2009; Moran, 2002). Additionally, an increasing number of defensive associations are being described where the symbionts protect their respective hosts and/or the host's food resources from parasites, pathogens or parasitoids (Currie *et al*., 1999; Kellner, 2002; Oliver *et al*., 2003; Kaltenpoth *et al*., 2005; Scarborough *et al*., 2005; Kroiss *et al*., 2010).

Members of the bacterial phylum Actinobacteria are especially prevalent as defensive symbionts due to their ecological and physiological prerequisites, including the ability to utilize a diverse range of nutritional resources and a remarkable versatility in producing secondary metabolites with antibiotic properties (Kaltenpoth, 2009). In contrast, direct evidence for nutritional mutualisms involving this bacterial group have been limited to vectors of the Chagas disease (*Rhodnius prolixus*) and their vitamin-supplementing *Rhodococcus* endosymbionts (Durvasula *et al*., 2008). Nonetheless, an increasing number of studies suggested Actinobacteria to be involved in the nutrition of a range of invertebrates. In scarab beetles of the genus *Pachnoda,* a number of bacterial strains with hemicellulolytic capabilities were isolated from the hindgut, including *Promicromonospora pachnodae*, an actinobacterial species capable of producing a range of xylanases and endoglucanases – two enzyme families involved in cellulose degradation (Cazemier *et al*. 1999, 2003; Andert *et al*. 2010). Additional studies have also

demonstrated the occurrence of Actinobacteria across different termite species. However, evidence for their nutritional contributions alongside indications of species-specific associations remains to be provided (Bignell *et al*., 1991; Shinzato *et al*. 2007). In this study, we present evidence for a highly specific partnership involving members of the Pyrrhocoridae insect family and two actinobacterial symbionts.

Within the Pyrrhocoridae, an oligophagous family of bugs, the best known members are cotton strainers of the genus *Dysdercus*, which are serious pests of cotton, and the red firebug (*Pyrrhocoris apterus*), an important model organism for endocrinology and physiology of hemimetabolous insects (Socha, 1993). Cotton stainers exhibit a cosmopolitan distribution mirroring that of the cotton cultivars, with each continent having its own group of species (Pearson, 1958; Ahmad and Schaefer, 1987). *Dysdercus* species harm cotton crops through the indelible staining of the cotton fiber resulting from the excrements of the bug or the accidental processing of insect-bearing bolls, as well as through the emanation of seed juices as a result of puncturing and feeding. Additionally, feeding by puncturing young cotton bolls usually results in the reduction of boll size (Pearson, 1958). *P. apterus* also specializes on seeds of the plant order Malvales, particularly dry seeds of linden trees (*Tilia cordata* and *T. platyphyllos*) (Socha, 1993; Kristenova *et al*., 2011). However, some studies also reported on the exploitation of seeds of other plants groups within and – to a lesser extent – beyond this plant order, as well as on the occasional feeding of firebugs on dead or weakened arthropods (Ahmad and Schaefer, 1987; Kershaw and Kirkaldy, 1908; Kristenova *et al*., 2011).

Previous studies investigating the microbial community of *P. apterus* revealed the presence of extracellular gut symbionts (*Coriobacterium glomerans*) belonging to the actinobacterial family Coriobacteriacae within the digestive tract of the insects, particularly in the M3 section (Haas and Konig, 1987; Kaltenpoth *et al*., 2009). Further

characterization of the whole microbiota of *P. apterus* using bacterial 16S rRNA amplicon pyrosequencing yielded an additional actinobacterial strain belonging to the Coriobacteriacae family (*Gordonibacter* sp.) alongside a range of other facultative and obligate anaerobes such as *Clostridium* sp., an undescribed Rickettsiales bacterium, *Klebsiella* sp. and *Lactococcus* sp. (Sudakaran *et al*., in press; Fig. 1). The consistency of the symbiotic microbial community across geographical localities and different food sources in *P. apterus* suggests that the complex microbiota might have co-evolved with the hosts over millions of years, and that they contribute significantly to the fitness of the insect (Sudakaran *et al*., in press).

Across different insect lineages, the mode of transmission of symbionts from one generation to another is highly variable. The majority of insects harboring intracellular primary symbionts rely on transovarial transmission modes where the infection originates inside of the female hosts during the early stages of oogenesis or embryogenesis (Buchner, 1965; Douglas, 1998; Schroder *et al*., 1996; Sauer *et al*., 2002; Nordon, 2006). In Hemiptera, however, post-hatch transmission is the most common mechanism of transfer, with symbiont acquisition resulting from ingestion of adult fecal droplets (Beard *et al*., 2002), through probing of symbiont containing capsules deposited close to the egg clutch (Fukatsu and Hosokawa, 2002; Hosokawa *et al*., 2005; 2006), or by egg-surface contamination and subsequent probing and uptake of the symbionts by the nymphs (larvae) during the early developmental stages (Prado *et al*., 2006). The latter route of symbiont transfer has also been described for Pyrrhocoridae, and surface sterilization of the egg surface resulted in symbiont-free individuals (Kaltenpoth *et al*., 2009).

In this study, by taking advantage of the transmission mechanism of the symbionts, we experimentally tested the significant contribution of the microbial community towards the fitness of *D. fasciatus* and *P. apterus*. We utilized a quantitative, community-level

analysis aimed at identifying strains that contribute directly to the overall fitness of their host. Furthermore, we assessed the specificity of this partnership in the two pyrrhocorid species by reciprocal exchange of symbionts.

3.3 RESULTS

3.3.1 Midgut microbiota of *D. fasciatus*

The midgut microbiota of *D. fasciatus* was analysed using 454 pyrosequencing of bacterial 16S rRNA amplicons. The sequencing data (10,026 sequences) revealed *Clostridium* sp. and *Lactococcus lactis* (Firmicutes), *C. glomerans* and *Gordonibacter* sp., (Actiobacteria), as well as several Gammaproteobacteria as the major microbial taxa in the gut of *D. fasciatus* (Fig. S1, Table S1). While the two Actinobacteria and the two Firmicutes are shared with *P. apterus* (Sudakaran *et al.,* in press), the Rickettsiales bacterium and *Klebsiella* sp, which are present in high frequencies in *P. apterus*, were not detected by 454 in *D. fasciatus*. Using diagnostic qPCR assays with primer pairs designed based on the *P. apterus* symbiont sequences, however, the two strains were consistently found in similar abundances across both bug species, indicating that the absence of these strains in the 454 dataset reflects a technical bias rather than true absence from *D. fasciatus*. In the case of *Klebsiella* sp., the inherently low taxonomic resolution of bTEFAP due to the short read lengths may cause the differences in the classification within Enterobacteriaceae between the *P. apterus* and the *D. fasciatus* symbionts, especially because this bacterial group contains many species (and even genera) with 16S rRNA similarities of more than 97% (our OTU clustering threshold similarity).

3.3.2 Success of symbiont manipulation procedure

Egg surface sterilization successfully eliminated *C. glomerans* in aposymbiotic treatments across both pyrrhocorid species as confirmed by *C. glomerans*-specific diagnostic PCRs (data not shown). In addition, the reinstitution of the microbial community using mid-gut suspensions from conspecific as well as heterospecific individuals confirmed that the nymphs readily accepted symbionts resulting from the experimental smearing of their egg surfaces.

Symbiont abundance estimates from quantitative PCR analyses of corresponding treatments for the two pyrrhocorid species (log-transformed) revealed that the core microbial communities of adult *P. apterus* and *D. fasciatus* were similarly influenced by prior surface sterilization of the eggs. Consistently affected were *C. glomerans* and *Gordonibacter* sp. abundances, with significant reductions in symbiont population sizes in the aposymbiotic treatments (ANOVA, $P < 0.05$) (Fig. 1 and 2). For both symbionts across the two host species, qPCR amplification in the aposymbiotic individuals was indistinguishable from background levels, and melting curve analyses suggested non-target amplification in late cycles (Fig. 1 and 2). Thus, the reported copy numbers likely represent overestimations of the two actinobacterial taxa, and aposymbiotic bugs may in fact be completely devoid of these symbionts.

The abundance of *Clostridium* sp. and *Lactococcus* sp. cells was significantly reduced by surface sterilization of the eggs only in *D. fasciatus* (ANOVA, $P < 0.05$) (Fig. 1), but not in *P. apterus* (Fig. 2). Uniformly unaffected by the egg sterilization procedures were the *Klebsiella* sp. and Rickettsiales populations, since their abundances were constant across treatments in both pyrrhocorid species (Fig. 4 and 5).

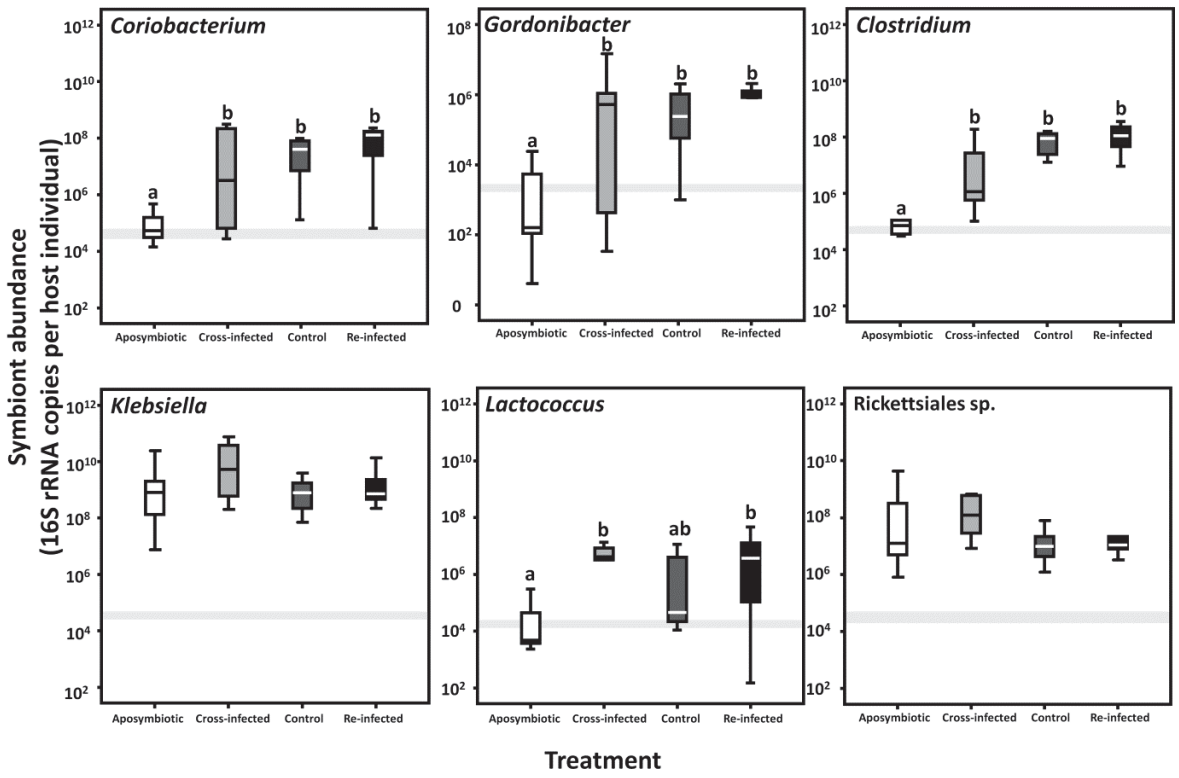

Figure 1. Quantitative PCR analyses of the six dominant microbial strains across the four experimental treatments for *D. fasciatus*. Symbiont numbers represent estimated 16S rRNA gene copy numbers obtained from qPCR assays. Shading of boxes signifies the experimental treatment. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Grey bands represent the range of unspecific background amplification for the negative controls. Different letters above boxes indicate significant differences in copy numbers (repeated-measures anova, $P<0.05$).

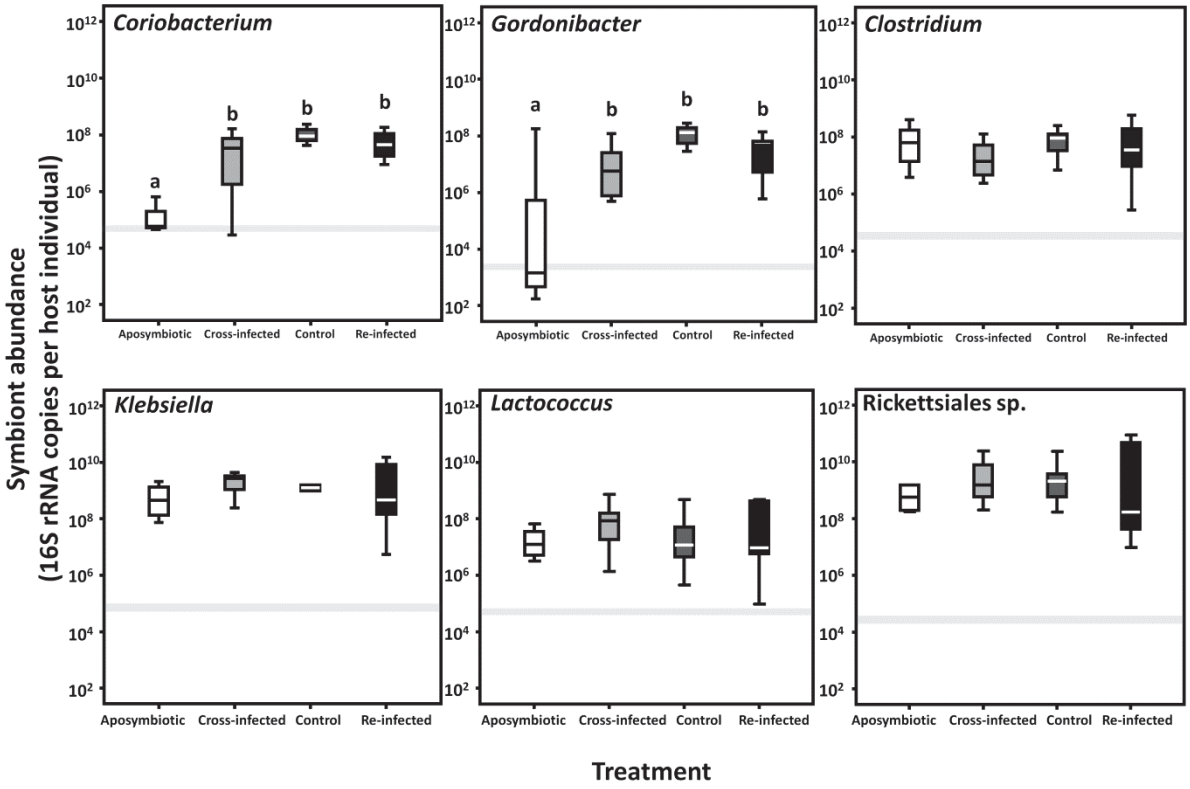

Figure 2. Quantitative PCR analyses of the six dominant microbial strains across the four experimental treatments for *P. apterus*. Symbiont numbers represent estimated 16S rRNA gene copy numbers obtained from qPCR assays. Shading of boxes signifies the experimental treatment. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Grey bands represent the range of unspecific background amplification for the negative controls. Different letters above boxes indicate significant differences in copy numbers (repeated-measures anova, $P<0.05$).

3.3.3 Fitness of *P. apterus* and *D. fasciatus* following symbiont manipulation

Aposymbiotic individuals across the two species of pyrrhocorids were found to suffer significantly higher mortality relative to the control treatments (Friedman test, $P < 0.01$ for both pairwise comparisons) (Fig. 3 A and B). Re-infection using mid-gut suspensions from conspecific individuals entirely rescued the adverse fitness effects caused by symbiont elimination. However, cross infection using mid-gut suspensions from heterospecific hosts did not significantly enhance chances to survive until adulthood

compared to aposymbiotic treatments (Fig. 3 A and B). The developmental time until adulthood was also found to be negatively influenced by symbiont elimination and exchange (Fig. 3 C) in *D. fasciatus* (Friedman test, $P < 0.05$). Similar trends were also observed for the *P.apterus* aposymbiotic treatment, but the effects were not significant (Fig. 3 D).

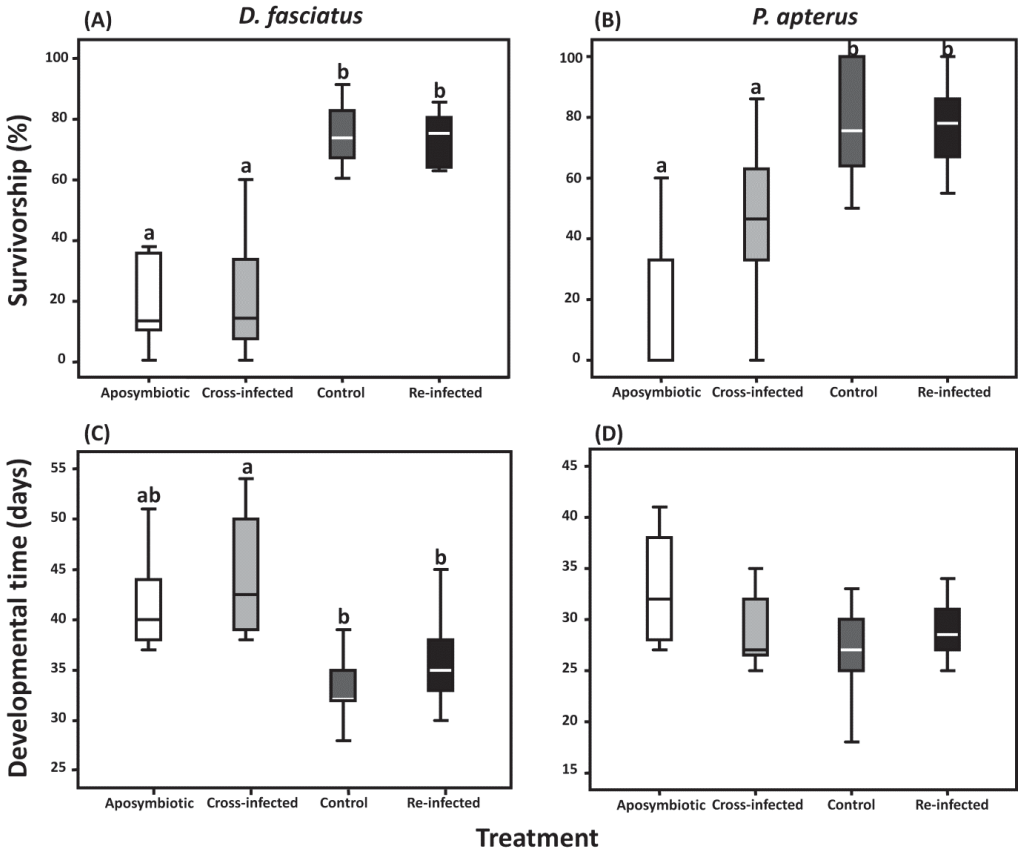

Figure 3. Fitness of aposymbiotic, symbiotic (control and re-infected) and cross-infected individuals of *D. fasciatus* (left panel) and *P. apterus* (right panel). **A** and **B**. Survivorship from egg hatching to adulthood. **C** and **D**. Nymphal development time until adult stage (days). Shading of boxes denotes the experimental treatment. Lines represent medians, boxes comprise the 25–75 percentile, and whiskers denote the range. Different letters above boxes indicate significant differences (Friedman test, $P<0.05$).

3.3.4 Reproductive success of adult *D. fasciatus* females following symbiont manipulation

On average, mating initiated across all symbiont-containing groups (control, re-infected and cross-infected) 3-7 days following the moult into adulthood. Behavioral assays revealed a complete lack of copulation among adults of the aposymbiotic treatment, which ultimately resulted in a lack of ovipositing and, thus, a significantly reduced reproductive success compared to the other treatments (ANOVA, $P < 0.05$) (Fig. 4 A). Aposymbiotic individuals were also observed to be less active, and the males were less aggressive in their mating pursuits.

For the control, re-infected, and cross-infected treatments, mated females laid on average 3.4 egg clutches throughout their lifespan, with the average reproductive output of 127-192 eggs. There were no differences in reproductive success across the three symbiont-containing treatments (Fig. 4 B) (ANOVA, $P > 0.05$).

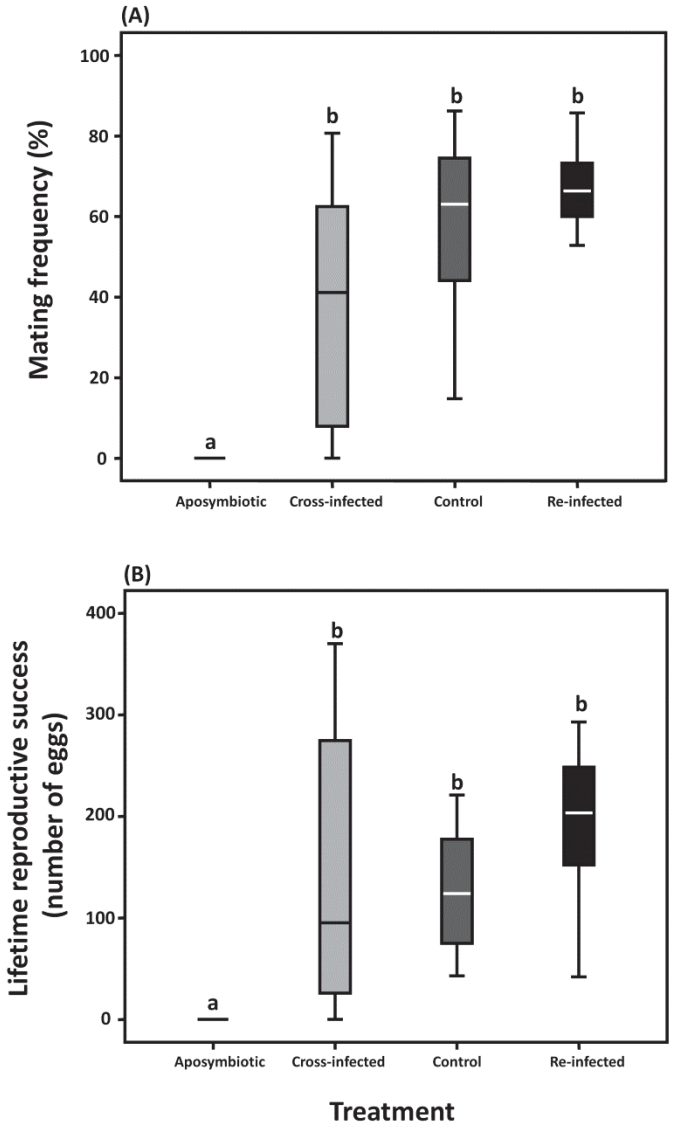

Figure 4. Reproductive success of aposymbiotic, symbiotic (control and re-infected) and cross-infected individuals of *D. fasciatus*. **A**. Mating frequencies. **B**. Lifetime reproductive success. Shading of boxes denotes the experimental treatment. Lines represent medians, boxes comprise 25–75 percentile, and whiskers denote the range. Different letters above boxes indicate significant differences (anova, $P<0.05$).

3.3.5 Correlations linking individual strain abundances to fitness

Abundance estimates for each of the six dominant strains across treatments were correlated with mortality of the respective replicate treatment groups to make inferences regarding the species-specific contributions of the symbionts towards the enhancement of host fitness. These analyses revealed positive correlations linking survivorship to higher frequencies of *C. glomerans* and *Gordonibacter* sp. (Spearman, $P < 0.05$) for both *P. apterus* and *D. fasciatus* (Fig. 5). While we are confident that the possible overestimation of actinobacterial abundances in aposymbiotic treatments constitutes a possible source of error that is conservative with regard to the hypothesis tested, we repeated the correlation analyses under the assumption that the aposymbiotic individuals were completely devoid of Actinobacteria as suggested by the melting curve analyses of the qPCRs. Hence, for this second analysis, we substituted the abundances of symbionts that yielded deviating melting curves in the qPCR assays and showed similar threshold cycle values as the negative controls with 0. The results remained the same, with abundances of *C. glomerans* and *Gordonibacter* sp. being significantly correlated with host fitness for both *P. apterus* and *D. fasciatus* (Spearman, $P < 0.05$). In addition to the Actinobacteria, *Clostridium* sp. abundances were positively correlated with survivorship in *D. fasciatus (*Spearman*,* $P < 0.001$) (Fig. S2), but not for *P. apterus* ($P = 0.677$) (Fig. S3). No significant correlations between survival and strain abundance were detected for any of the other strains (Spearman, $P > 0.05$ for all correlations, Fig. S2 and S3).

Mortality breakdown per developmental stage among aposymbiotic individuals of *P. apterus* indicated that mortality is highest between the 2nd and 4th instars (Fig. 6 A), which correlates directly to the host developmental stages where, according to quantitative PCR analyses by Sudakaran *et al.* (in press), the gut microbial community (including the Coriobacteriacae symbionts) exhibits the highest growth rates (Fig. 6 B).

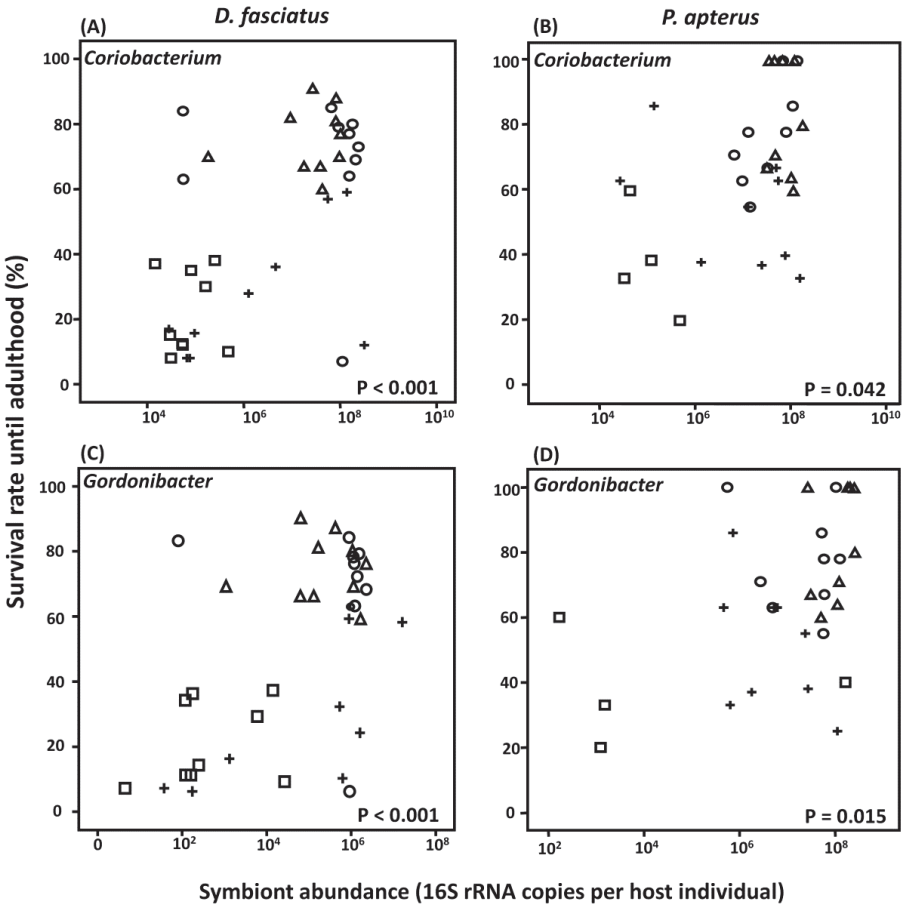

Figure 5. Correlations of survival rate and Coriobacteriacae abundances for *D. fasciatus* (A, C) and *P. apterus* (B, D) across treatments. Survival (%) relates to the survival rate of replicate treatments from egg hatching to adulthood. Coloration of samples signifies the experimental treatment each individual was ascribed to (square = aposymbiotic, cross = cross infected, triangle = untreated control, circle = re-infected). Lines denote significant correlations of symbiont copy number and survival rate (Spearman, $P < 0.05$).

3.3.6 Fitness of *D. fasciatus* fed on sunflower seeds

To assess the possible effects of specific toxic components in the linden seed diet on the fitness of symbiotic and aposymbiotic bugs, we performed an additional experiment with sunflower seeds that are devoid of the toxic compounds often present in plants of the order Malvales. As on the linden seed diet, however, aposymbiotic individuals of *D. fasciatus* that were fed exclusively on sunflower seeds were found to suffer significantly higher mortality relative to the control treatment (Wilcoxon signed ranks test, $P < 0.05$) (Fig. 7 A). Additionally, developmental time until adulthood was significantly increased after symbiont elimination in *D. fasciatus* (Friedman test, $P < 0.05$, Fig. 7 B). Interestingly, both aposymbiotic and symbiotic sunflower-fed bugs exhibited higher mortality and longer developmental times than their linden seed-fed counterparts, respectively (Fig. 3 and Fig. 7).

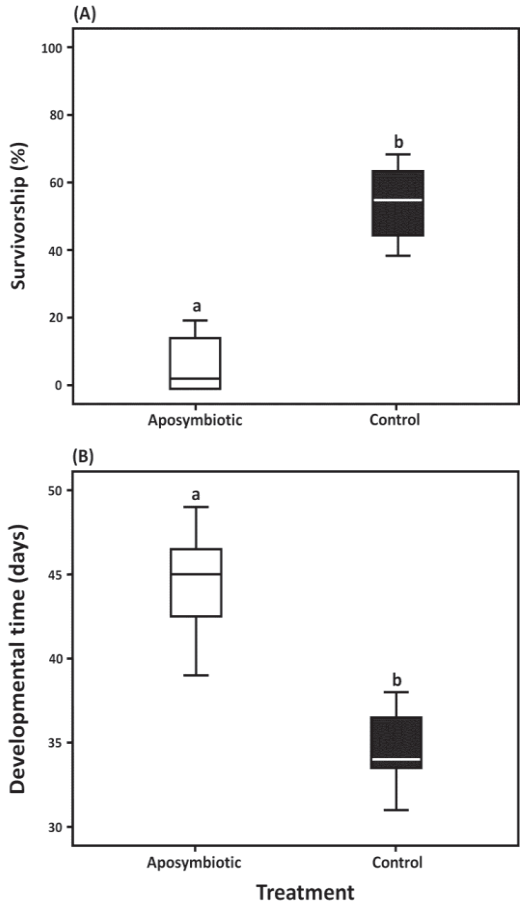

Figure 6. Fitness of aposymbitic and control groups for D. fasciatus when fed on sunflower seeds. (**A**) Survival rate from egg hatching to adulthood. (**B**) Nymphal development time until adult stage (days). Boxes represent the 25-75 percentile, the middle bands represent the median, and the whiskers denote the range. Different letters above boxes indicate significant differences (Wilcoxon test, $P < 0.05$).

3.4 DISCUSSION

The ubiquity of Actinobacteria in the environment, coupled with their capacity towards producing substances with antimicrobial compounds has probably predisposed them to engage in defensive symbioses with soil-dwelling insects (Kaltenpoth, 2009). In contrast, direct evidence for their involvement in nutritionally-based partnerships has been relatively scarce (but see Lake and Friend, 1967; 1968). Here we report on the occurrence of two actinobacterial taxa (*C. glomerans* and *Gordonibacter* sp.) as essential nutritional symbionts in pyrrhocorid bugs.

Fitness assays demonstrate that symbiont elimination via surface sterilization of the egg surface significantly reduces host fitness and reproductive output of both pyrrhocorid species used in this study, which is consistent with the putative function of the symbionts for the nutritional upgrading of the host. The ability to restore normal fitness to the resulting nymphs from previously sterilized eggs by reapplying native microbial suspensions ensures that the method of symbiont elimination was not responsible for the adverse effects observed for aposymbiotic individuals (Fig. 3). Consistent with these findings is the breakdown of aposymbiotic treatment mortality per developmental stage. These analyses point towards the 2nd, 3rd and 4th instars as the stages where individuals from this treatment suffer the greatest reduction in fitness (Fig. 6 A). It is during those stages that the Coriobacteriacae symbiont populations experience the highest growth rate in untreated control individuals (Fig. 6 B), further confirming the strong correlation between actinobacterial presence and fitness effects. This is congruent with a previous study addressing the contributions of the actinomycete *Rhodococcus rhodnii* towards its triatomine host (*Rhodnius prolixus*) where aposymbiotic individuals suffered the greatest mortality during the 3rd and 4th instars (Lake and Friend, 1968).

The combined analysis of the 454 pyrosequencing and qPCR revealed that the core microbial community, namely Actinobacteria (*C. glomerans* and *Gordonibacter* sp.), Firmicutes (*Clostridium* sp., and *Lactococcus lactis*) and Proteobacteria (an uncultivated bacteria associated with the Rickettsiales, and *Klebsiella* sp.) is shared across both pyrrhocorid hosts (Fig. S1). The symbiont manipulation and exchange procedure utilized in our study consistently influenced the microbial community present within pyrrhocorid bugs. For both bug species, we observed significant variation between aposymbiotic and control treatments for *Gordonibacter* sp. and *C. glomerans* frequencies, suggesting that that the egg surface sterilization procedure was especially effective in ridding pyrrhocorids from their Coriobacteriacae symbionts (Fig. 1 and 2). More direct evidence for strain-specific contributions to host fitness was provided by the correlative analyses linking estimated strain abundances for emerging adults to the survivorship of the replicate treatment that the individual belonged to. These correlations singled out *Gordonibacter* sp. and *C. glomerans* as essential symbionts given their consistently significant correlations across the two pyrrhocorid species (Fig. 5). This indicates that one or both actinobacterial strains are important for the fitness of pyrrhocorid bugs. However, until strain-specific reinfection procedures are performed, we cannot disentangle the individual contributions of *C. glomerans* and *Gordonibacter* sp. towards host fitness. Despite observing a significant positive correlation between the frequency of *Clostridium* sp. and survivorship in *D. fasciatus*, their mutualistic potential was undermined by their high abundance values in the aposymbiotic treatments of *P. apterus* (Fig. S2 and S3). Additionally, no such tends were observed for *Lactococcus* sp. in either pyrrhocorid species despite an apparent fluctuation in symbiont abundances in *D. fasciatus* as a result of the surface sterilization procedure (Fig. S2). Possibly, the infection with *Clostridium* sp. and *Lactococcus* sp. may be beneficial for pyrrhocorids, but not essential for growth

and development of the host. The consistency of both proteobacterial species (*Klebsiella* sp. and the undescribed Rickettsiales bacterium) across all treatments suggests that those symbionts are not influenced by the surface sterilization procedure (Fig. 1 and 2), possibly due to a transovarial transmission route where the infection of the eggs can originate inside of the female host during the early stages of oogenesis. Due to our experimental approach targeting only bacteria that are transmitted via the egg surface, we cannot make any inferences here about the influence of these strains on host fitness.

High mortality rates observed for pyrrhocorid bugs that were cross-infected with microbial suspensions from heterospecific individuals indicate that the fitness benefits conferred to the insect host are governed by a high degree of specificity. Interestingly, quantitative PCR measurements of cross-infected individual indicate that the acquired number of Coriobacteriacae symbiont cells (1.4×10^7 cells) were comparable to those present in control and re-infected treatments (Fig 1 and 2). Thus, host-symbiont interactions rather than lower symbiont titers appear to be responsible for the fitness reductions in cross-infected bugs. This is contrary to the experimental exchange of obligate gut symbiotic bacteria of the stinkbugs *Megacopta punctatissima* and *Megacopta cribraria*, where symbionts from heterospecific hosts were found to fully restore fitness irrespective of the receiving insect host species (under the condition that the insects were fed on optimal host plants) (Hosokawa *et al*., 2007). The high degree of relatedness of *M. punctatissima* and *M. cribraria* can account for the variation in symbiont specificity observed in Hosokawa *et al*. 2007, where symbiont exchange was conducted on an inter-species level as opposed to the inter-generic scale of this study.

Reports on the feeding biology of Pyrrhocoridae have indicated a clear preference for ripened seeds of plants belonging to the angiosperm order Malvales (including cotton) (Socha, 1993; Ahmad and Schaefer, 1987). However, some studies have also pointed

towards limited intra- and interspecific carnivorous behavior where the bugs have been found to utilize their stylets to attack slower, weaker prey or recently deceased arthropods (Ahmad and Schaefer, 1987; Kershaw and Kirkaldy, 1908). Therefore, unlike symbiotic relationships in insects persisting exclusively on nutritionally deficient diets (e.g. blood or sap-feeding insects) - but similar to the *Blochmania*-harboring carpenter ants (Feldhaar *et al*., 2007) – pyrrhocorids, to a certain degree, appear to supplement their specialized food source of Malvales seeds with a nutritionally rich carnivorous diet. This raises the question regarding the putative function of the Coriobacteriacae symbionts in this family.

Generally, bacterial symbionts can enable insect hosts to exploit specialized food sources by supplying additional nutrients that are limited in their diet, assisting in the degradation of complex plant tissue, or by providing a detoxifying function against the plant's secondary compounds (Douglas, 2009). Cotton plants and a range of other Malvaceae species contain high concentrations of gossypol, a phenolic aldehyde that acts as an inhibitor for several dehydrogenase enzymes (Abou-Donia, 1976; Reeves and Valle, 1923), as well as malvalic and sterculic acid, which are cyclopropenoic fatty acids that have been demonstrated to inhibit the desaturation of stearic acid in animals fed on cotton-seed derivatives (Allen *et al*., 1967). However, pyrrhocorid bugs as well as some other herbivorous insects appear to have evolved a mechanism to cope with the toxic plant compounds. We hypothesized that the actinobacterial symbionts of Pyrrhocoridae may aid in the detoxification of gossypol or other toxic components of Malvales seeds. However, our current findings do not support this hypothesis, since aposymbiotic individuals exhibited high mortality even when fed on a gossypol and cyclopropenoic acid-free diet composed of sunflower seeds (Fig. 7). Thus, the primary role of the symbionts appears to be a more general nutritional function rather than the detoxification of noxious secondary plant compounds. Whitsitt (1933) demonstrated that cottonseed

meals offer prohibitively low amounts of vitamins, especially riboflavin, to be utilized exclusively as a food source. Based on these considerations and the results of the present study, it seems likely that the main function of the Coriobacteriaceae symbionts lies either in the degradation of complex plant compounds (e.g. cellulose) or the supplementation of the diet with limiting vitamins. However, until we assess the importance of the gammaproteobacterial symbionts (*Klebsiella* sp. and the Rickettsiales bacterium) and Firmicutes (*Clostridium* sp. and *Lactococcus* sp.), and disentangle the individual contributions of *C. glomerans* and *Gordonibacter* sp. through strain specific reinfections, we cannot rule out detoxification as a putative additional function of the microbial community.

Members of the genus *Dysdercus* are of serious importance given their worldwide distribution spanning every major cotton-producing continent, and the irreversible damage that they confer to this economically important crop plant. Elucidating the contribution of the resident symbiotic community to the fitness of pyrrhocorid bugs and the fundamental mechanisms by which these benefits are conferred not only expands our understanding of the ecology of a serious agricultural pest, but may also provide novel leads for biological control by manipulation of the host's microbiota. To elucidate the exact functions of *C. glomerans* and *Gordonibacter* sp. towards their hosts, additional approaches examining the metabolism, physiology and genomic signatures of these symbionts are required.

While most studies investigating insect-bacterial symbioses are focused on elucidating the contributions of a single microbial symbiont towards its host (Douglas, 1998; 2009; Moran, 2002), the majority of insects, including the Pyrrhocoridae insect family, are inhabited by a complex gut microbial community (Buchner, 1965; Sudakaran *et al*., in press; Dillon and Dillon, 2004; Ferrari and Vavre, 2011). Despite their abundance and

ecological importance, functional analyses detailing the fitness contributions of symbionts in multipartite interactions have been scarce to date. Thus, we believe that our quantitative community-level approach for investigating the fitness contributions of several bacterial strains towards a single host presents a powerful tool that can be broadly utilized to gain important insights into functional roles of individual microbial taxa in symbiotic systems that involve multiple partners.

3.5 EXPERIMENTAL PROCEDURES

3.5.1 Insect sampling and rearing

Adult specimens of *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae) were collected from the vicinity of linden trees (*Tilia cordata* and *Tilia platyphyllos*) in Jena, Germany. The insects were reared in plastic containers (20×35×22 cm) at a constant temperature of 28°C and long light regimes (16h/8h light/dark cycles) to prevent the insects from entering into diapausal states. Bugs were provided with previously autoclaved water and crushed dry linden seeds (*T. cordata* and *T. platyphyllos*). *Dysdercus fasciatus* were acquired from a laboratory culture maintained at the University of Würzburg, Germany, which had been originally collected from the Comoé National Park, Côte d'Ivoire. The insects were reared under the same conditions as described for *P. apterus.*

3.5.2 Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) and data analysis

Prior to DNA extraction, six complete *D. fasciatus* adults (three males and three females) were submerged in liquid nitrogen and crushed with sterile pestles. DNA was extracted

using the MasterPure^{TM} DNA Purification Kit (Epicentre Technologies) according to the manufacturer's instructions. BTEFAP was done using an external service provider (Research and Testing Laboratories, Lubbock, USA) with 16S rRNA primers Gray28F and Gray519R (Ishak *et al.* 2011; Sun *et al.* 2011). A sequencing library was generated through one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar high fidelity *Taq* polymerases (Qiagen). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Research and Testing Laboratory (RTL, Lubbock, TX, USA, http://www.researchandtesting.com). All low quality reads (quality cut-off = 25) and sequences < 200 bp were removed following sequencing, which left 10,026 sequences for analysis. The raw reads (sff files) were deposited in the short read archive (SRA) of NCBI under accession number SRA058953.

Analysis of the high-quality reads was conducted by using QIIME (Caporaso *et al.* 2010b). Cdhit (Li and Godzik 2006) and Uclust (Edgar 2010) with 97% similarity cut-offs was employed in Multiple OTU picking to cluster the sequences into operational taxonomic units (OTUs). The most abundant sequences was chosen as representative sequence was for each OTU picked and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso *et al.* 2010a), with the minimum sequence identity percent set to 75%. RDP classifier was used for taxonomy assignment (Wang *et al.* 2007), with a minimum confidence to record assignment set to 0.80. OTU tables was generated describing the occurrence of bacterial phylotypes within the sample. The table was then manually curated by removing low-frequency reads (<0.1% in all samples) and through database comparisons of the representative sequences with the NCBI and RDP databases. Based on the BLASTn results, OTUs with the same genus-level assignments were combined for visualization of the results. The revised OTU table

was used to construct heatmaps using the MultiExperiment Viewer (MeV) software (Saeed *et al.* 2003).

3.5.3 Experimental manipulation of the microbial gut community

To avoid pseudoreplication, ten egg clutches (>35 eggs each) from different females of *P. apterus* and *D. fasciatus,* respectively, were harvested five days after ovioposition and kept separately. Each clutch was randomly separated into four different experimental treatments: (1) aposymbiotc, (2) re-infected with native microbial community, (3) cross-infected with heterospecific microbial community, (4) untreated control. Aposymbiotic individuals were generated by surface sterilization of eggs following the procedure describe by Prado *et al*. [28]. Briefly, eggs were submerged in bleach (12% NaOCl) for 45 seconds, followed by a five minute 95% ethanol treatment which was subsequently washed off thoroughly with sterile H_2O. Reapplication of the symbiotic microbial community was accomplished by spreading a suspension of the crushed M3 gut region of a conspecific or a heterospecific individual over previously sterilized eggs. Future contamination of the experimental treatments with environmental bacteria was reduced by using autoclaved food (linden seeds) and water throughout the course of the study.

To investigate the possibility of symbiont-mediated detoxification of plant secondary compounds associated with the linden seeds, eight additional egg clutches from different *D. fasciatus* females were harvested and separated into two different experimental treatments: (1) aposymbiotic, and (2) untreated control. Both treatments were reared as described above, but the newly hatched nymphs were supplemented with autoclaved sunflower seeds instead of linden seeds.

3.5.4 Fitness measurements

Individuals across all experimental treatments were observed on a daily basis for the assessment of fitness effects across the different groups. Growth rate (nymphal stage) and survival until adulthood (%) were recorded. A replicate treatment group of bugs was defined to have completed a nymphal stage when 50% of the nymphs had successfully molted into the following developmental stage.

To quantify the reproductive success of emerging adult *D. fasciatus* females, eight females and eight males were collected across the replicates of each treatment, respectively. Due to the high mortality in the aposymbiotic group, only six males could be allocated to the eight aposymbiotic females. For each female, we measured the copulation frequencies and the total number of eggs laid throughout its lifespan (lifetime reproductive success). Mating frequencies were determined by assessing the percent of days an individual was found in a mating pair. All egg masses laid by each of the females were collected, and the number of eggs was counted throughout the lifespan of the bug.

3.5.5 DNA extraction and PCR screening for *C. glomerans*

A single emerging adult individual from every experimental treatment for both species was subjected to DNA extraction three days after adult emergence (Kaltenpoth *et al*., 2009). Males and females were used indiscriminately, as they have been shown previously to harbor identical microbial communities in the mid-gut (Sudakaran *et al*., in press). To validate that the surface sterilization and reinfection procedures were successful, primers specific for *C. glomerans* (Kaltenpoth *et al*., 2009) were utilized to screen for the symbiont using diagnostic PCR reactions (Table 1). To account for any failures during DNA extraction, additional PCR screens targeting the host 18S rRNA

genes of *P. apterus* and *D. fasciatus* were performed (Li *et al*., 2005), and negative samples were discarded from further analysis (Table 1). PCR amplifications were conducted on a VWR® Gradient Thermocycler (VWR, Radnor, PA, USA) using 12.5 µl reactions, including 1 µl of DNA template, 1×PCR buffer (20 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 mM dNTPs, 0.8 µM of each primer, and 0.5 µl of Taq DNA polymerase (VWR, Radnor, PA, USA). The following cycling parameters were used: 3 min at 94ºC, followed by 32 cycles of 94ºC for 40 s, 68/66ºC for 1 min (Cor/Pyr primers, respectively), and 72ºC for 1 min, and a final extension time of 4 min at 72ºC.

Table 1. Diagnostic primers used for the specific detection of *C. glomerans* in pyrrhocorids and for positive control amplification of host DNA.

Primer	Primer sequence (5′–>3′)	Orientation	Target group	Reference
Cor_2F	GGTAGCCGGGTTGAGAGACC	Fwd.	*C. glomerans*	Kaltenpoth *et al*., 2009
Cor_1R	ACCCTCCCMTACCGGACCC	Rev.		
Pyr18S_2F	GGGAGGTAGTGACAAAAAATAACG	Fwd.	Pyrrhocoridae	Sudakaran *et al*., in press
Pyr18S_4R	GTTAGAACTAGGGCGGTATCTG	Rev.		

3.5.6 Quantitative PCR

Quantitative PCRs (qPCRs) for the six dominant bacterial strains within pyrrhocorids (Sudakaran *et al*., in press) were conducted across the four experimental treatments of *P. apterus* and *D. fasciatus* using a RotorGene®-Q cycler (Qiagen, Hilden, Germany), with the same individual DNA extracts used for the *C. glomerans* screen. The final reaction volume of 25 µl included the following components: 1 µl of DNA template, 2.5 µl of each primer (10 µM), 6.5 µl of autoclaved distilled H_2O, and 12.5 µl of SYBR Green Mix (Qiagen, Hilden, Germany). The primers used were specific for the 16S rRNA genes of *C. glomerans, Gordonibacter* sp., *Clostridium* sp., the undescribed Rickettsiales bacterium, *Klebsiella* sp. and *Lactococcus lactus* (Table 2) (Sudakaran *et al*., in press). Verification of primer specificity was conducted *in silico* by comparison with reference sequences of all bacterial taxa in *P. apterus* (Sudakaran *et al*., in press). Additionally, PCR products of all strain-specific PCRs were sequenced without prior cloning from mid-gut samples of *P. apterus* (Sudakaran *et al*., in press) and *D. fasciatus* (this study) to confirm primer specificity *in vitro*. Conditions for qPCR were optimized using a VWR® Gradient Thermocycler (VWR, Radnor, PA, USA) at various annealing temperatures (60-68 °C). Standard curves (10-fold dilution series from 1 ng/µl to 10^{-6} ng/µl) were generated using purified PCR products for all six primers after measuring the PCR products using a NanoDropTM1000 spectrophotometer (Peqlab). The following cycling parameters were used: 95°C for 10 min., followed by 45 cycles of 68ºC for 30 s, 72ºC for 20 s, and 95ºC for 15 s. Subsequently, a melting curve analysis was conducted by increasing the temperature from 60ºC to 95ºC within 20 min. Six replicates of one of the standard concentrations were used, for each primer pair and concentration, for the configuration and calibration of the standard curve. The resulting averages were then utilized to correct for possible errors in the DNA concentration measurments. Based on

the standard curve, absolute copy numbers of specific 16S templates were calculated according to Lee *et al.* (Lee *et al*., 2006; 2008).

Table 2. Bacterial 16S rRNA primers used for the strain-specific qPCR assays.

Primer	Primer sequence (5′–>3′)	Orientation	Target group	Reference
Cor_2F	GGTAGCCGGGTTGAGAGACC	Fwd.	*C. glomerans*	Kaltenpoth *et al*., 2009
Cor_1R	ACCCTCCCMTACCGGACCC	Rev.		
fD1 (=27F)	AGAGTTTGATCCTGGCTCAG	Fwd.	*Gordonibacter* sp.	Weisburg *et al*., 1991
Egg_1R	CCGGAGCTTCTTCTGCAGGT	Rev.		this study
Proteobac_16s_fwd	GTGGCAAACGGGTGAGTAAT	Fwd.	Undescribed Rickettsiales species	Sudakaran *et al*., in press
Proteobac_16s_rev	GAAGTCTGGGCCGTATCTCA	Rev.		
Klebsiella_250-fwd	CAGCCACACTGGAACTGAGA	Fwd.	*Klebsiella* sp.	Sudakaran *et al*., in press
Klebsiella_453-rev	GTTAGCCGGTGCTTCTTCTG	Rev.		
Clostridium_1050-fwd	CTCGTGTCGTGAGATGTTGG	Fwd.	*Clostridium* sp.	Sudakaran *et al*., in press
Clostridium_1248-rev	GCTCCTTTGCTTCCCTTTGT	Rev.		
Lactococcus_975-fwd	CGCTCGGGACCTACGTATTA	Fwd.	*Lactococcus lactus*	Sudakaran *et al*., in press
Lactococcus_1175-rev	GCAGCAGTAGGGAATCTTCG	Rev.		

3.5.7 Statistical analysis

For both Pyrrhocoridae species, growth and survival rates until adulthood were compared across the four experimental treatments using Friedman tests with Wilcoxon-Wilcox posthoc measures for the analysis of dependent samples (with replicates across treatments paired via individual egg clutches), using the statistical software BiAS 7.40 (Epsilon-Verlag; Hochheim-Darmstadt, Germany). To compare symbiont 16S copy numbers estimated in the qPCRs, and reproductive success of emerging females (only *D. fasciatus*), ANOVA was used as implemented in the SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA).Growth and survival rates of the two *D. fasciatus* treatments (aposymbiotic and untreated control) reared on sunflower seeds were compared using Wilcoxon signed ranks test using the BiAS 7.40. Non-parametric bivariate correlations (Spearman) between symbiont abundance (of the representative individual subjected to qPCR) and treatment mortality within each replicate were performed for each symbiont strain in order to infer the individual contributions of the strains towards the host's overall fitness and development (SPSS 17.0).

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3.8 SUPPLEMENT

Table S1. Absolute and relative (in percent) sequence abundance of bacterial OTUs in *D. fasciatus*.

Bacterial taxa	Sequence abundance	Relative abundance
Clostridium (Firmicutes)	1998	19.9
Gordonibacter (Actinobacteria)	1011	10.1
Coriobacterium (Actinobacteria)	711	7.09
Lactococcus (Firmicutes)	2085	20.8
Enterobacter (Proteobacteria)	1455	14.5
Stenotrophomonas (Proteobacteria)	983	9.80
Kluyvera (Proteobacteria)	720	7.18
Lactobacillus (Firmicutes)	699	6.97
Commensalibacter (Proteobacteria)	71	0.70
Unknown Actinomcyetes	50	0.49
Azospirillum (Proteobacteria)	46	0.46
Agrobacterium (Proteobacteria)	39	0.39
Brevundimonas (Proteobacteria)	34	0.34
Methylobacterium (Proteobacteria)	34	0.34
Sphingomonas (Proteobacteria)	25	0.25
Enterococcus (Firmicutes)	24	0.24
Variovorax (Proteobacteria)	14	0.14
Bartonella (Proteobacteria)	13	0.13
Propionibacterium (Actinobacteria)	13	0.13
Hafnia (Proteobacteria)	1	0.01
Total	**10026**	**100**

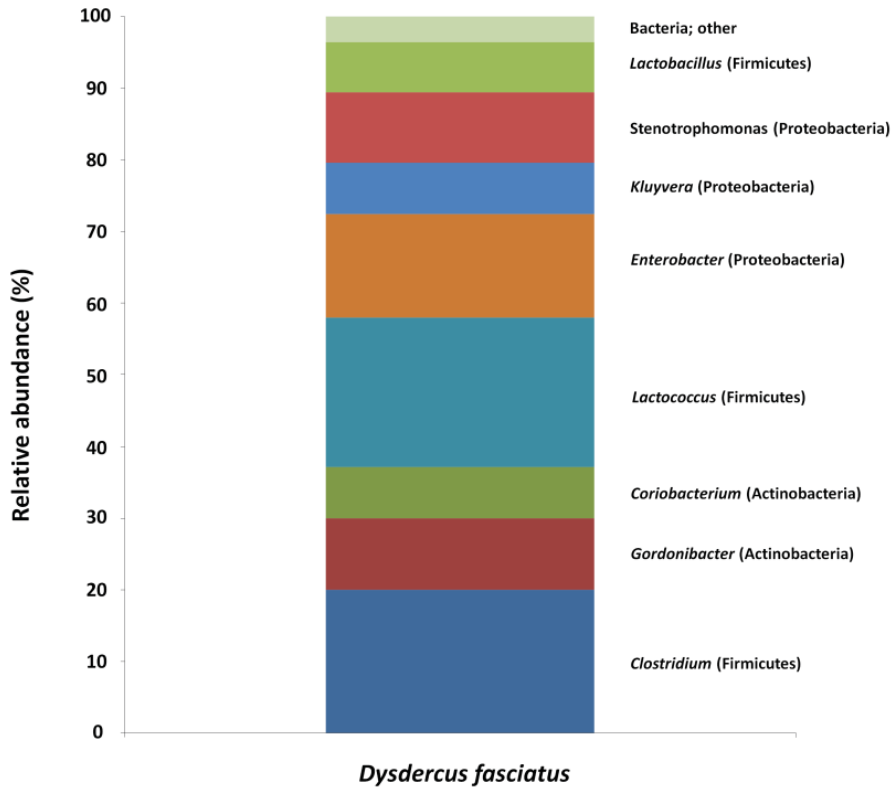

Figure S1. Bacterial community of *P. apterus* and *D. fasciatus*. Relative abundance of gut bacterial taxa (genus-level) from 454 pyrosequencing of 16S rRNA amplicons (10,026 reads in total) of a pooled sample of six adult individuals.

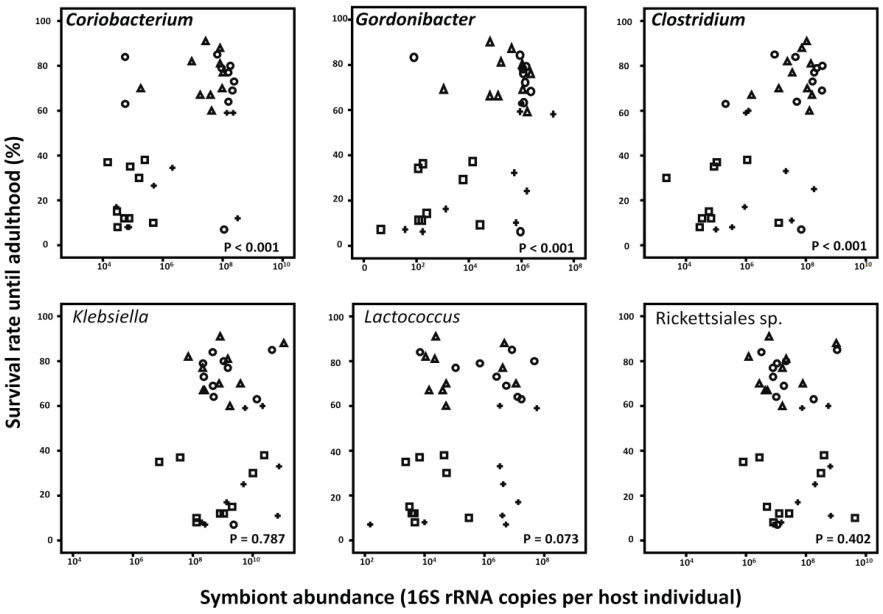

Figure S2. Correlations of survival rate and individual symbiont abundances for *D. fasciatus* across treatments. Survival (%) relates to the survival rate of replicate treatments from egg hatching to adulthood. Shapes of data points signifies the experimental treatment each individual was ascribed to (square = aposymbiotic, cross = cross infected, triangle = untreated control, circle = re-infected). Bold subtitles denote significant correlations of symbiont copy numbers and survival rates (Spearman, $P < 0.05$).

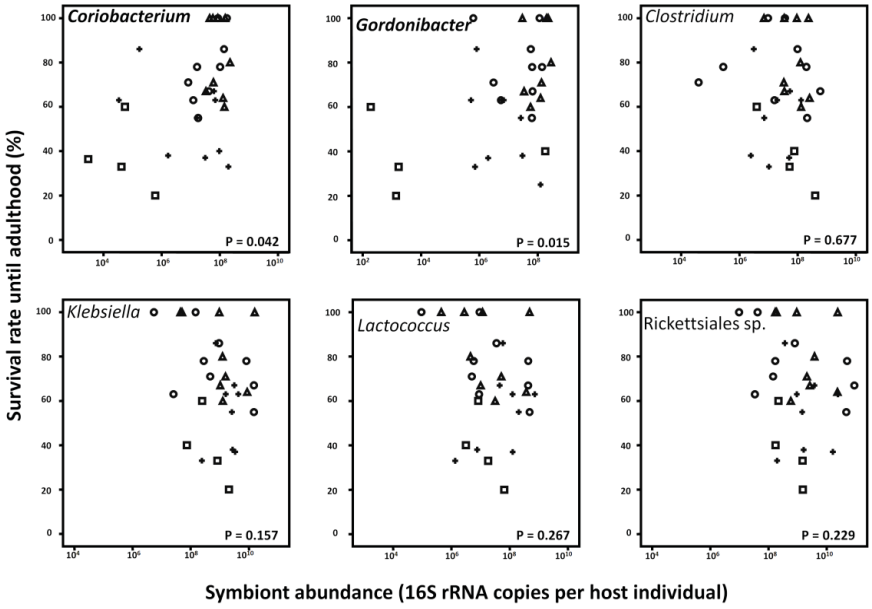

Figure S3. Correlations of survival rate and individual symbiont abundances for *P. apterus* across treatments. Survival (%) relates to the survival rate of replicate treatments from egg hatching to adulthood. Shapes of data points signifies the experimental treatment each individual was ascribed to (square = aposymbiotic, cross = cross infected, triangle = untreated control, circle = re-infected). Bold subtitles denote significant correlations of symbiont copy numbers and survival rates (Spearman, $P < 0.05$).

CHAPTER 4

Evolutionary transition in symbiotic syndromes enabled diversification of phytophagous insects on an imbalanced diet.

CHAPTER 4

Evolutionary transition in symbiotic syndromes enabled diversification of phytophagous insects on an imbalanced diet.

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ISME (in press)

4.1 ABSTRACT

Evolutionary adaptations for the exploitation of nutritionally challenging or toxic host plants represent a major driving force behind the diversification of phytophagous insects. Although symbiotic bacteria are known to play essential nutritional roles for insects, examples of adaptive radiations into novel ecological niches following the acquisition of specific symbionts remain scarce. Here we characterized the microbiota across bugs of the family Pyrrhocoridae and investigated whether the acquisition of vitamin-supplementing symbionts enabled the hosts' adaptive radiation on the nutritionally

imbalanced and chemically well-defended seeds of Malvales plants as a food source. Our results indicate that vitamin-provisioning Actinobacteria (*Coriobacterium* and *Gordonibacter*), as well as Firmicutes (*Clostridium*) and Proteobacteria (*Klebsiella*) are widespread across Pyrrhocoridae, but absent from the sister family Largidae and other outgroup taxa. Despite the consistent association with a specific microbiota, the Pyrrhocoridae phylogeny is neither congruent with a dendrogram based on the hosts' microbial community profiles nor phylogenies of individual symbiont strains, indicating frequent horizontal exchange of symbiotic partners. Phylogenetic dating analyses based on the fossil record reveal an origin of the Pyrrhocoridae core microbiota in the late Cretaceous (81.2-86.5 mya), following the transition from crypt-associated beta-proteobacterial symbionts to an anaerobic community localized in the M3 region of the mid-gut. The change in symbiotic syndromes (i.e. symbiont identity and localization) and the acquisition of the pyrrhocorid core microbiota coincided with the evolution of their preferred host plants (Malvales), suggesting that the symbionts enabled their hosts to successfully exploit this imbalanced nutritional resource and subsequently diversify in a competition-poor ecological niche.

4.2 INTRODUCTION

The evolutionary success of herbivorous insects and their diversification into a wide range of ecological niches is closely connected to the diversification of their host plants (Ehrlich and Raven 1964). Herbivores and plants engage in an evolutionary arms race, with plants continuously evolving novel chemical defenses or imbalanced nutritional composition to reduce herbivore attacks, and insects adapting by developing strategies to circumvent defenses and nutritional challenges. Thus, the diversification of terrestrial plants opened up a multitude of ecological niches, permitting the adaptive radiation of herbivorous insects (Farrell and Mitter 1994). A famous example are the monarch butterflies that evolved the ability to detoxify or sequester protective cardiac glycosides produced by plants of the milkweed and dogbane families (Asclepiadaceae and Apocynaceae) and thereby successfully colonize these host plants and diversify into about 150 species (Ehrlich and Raven 1964).

However, host plant selection and exploitation as a nutritional resource are not only determined by the metabolic capabilities of the insects themselves, but also their associated microbiota (Douglas 2009, Hosokawa *et al* 2007). Microbial symbionts can confer important ecological traits to their hosts, including contributions to digestion (Breznak and Brune 1994, Lundgren and Lehman 2010, Warnecke *et al* 2007), detoxification (Dowd 1989, Genta *et al* 2006), and nutrient provisioning (Borkott and Insam 1990, van Borm *et al* 2002). Such symbiotic interactions can play a crucial role in the evolutionary diversification of herbivorous insects by facilitating expansion into novel ecological niches (Janson *et al* 2008, Moran 2007). Accordingly, expansion of the host plant range and/or increased diversification have been observed in gall midges after the acquisition of fungal symbionts (Joy 2013), and in aphids after the acquisition of facultative symbiont *Regiella insecticola* (Tsuchida *et al* 2011). Furthermore, the

replacement of an ancestral beta-proteobacterial symbiont with *Baumannia* in sharpshooters (Cicadellidae) enabled the hosts to shift from using phloem sap as main nutrient source to the even less nutritionally balanced xylem sap (Takiya *et al* 2006). However, despite the wealth of information that is available on the benefits microbes can provide to their insect hosts, the role of symbionts in driving the diversification of insects and their expansion into novel ecological niches remains less well understood (Janson *et al* 2008).

Within the megadiverse insect order Hemiptera, the infraorder Pentatomomorpha (i.e. the '*stinkbugs*') contains over 12,500 species (Henry 1997, Schaefer 1993, Schuh and Slater 1995), many of which harbour beneficial symbionts that contribute significantly to host fitness (Abe *et al* 1995, Fukatsu and Hosokawa 2002, Huber-Schneider 1957, Kikuchi *et al* 2009, Muller 1956, Salem *et al* 2013, Schorr 1957, Tada *et al* 2011). Interestingly, symbiotic syndromes (i.e. identity and localization of the symbionts) vary greatly among Pentatomomorpha, indicating frequent transitions during the evolutionary history of this group. The most common symbiont-bearing organs across the superfamilies Lygaeoidea, Coreoidea, and Pentatomoidea are specialised sacs or tubular outgrowths, called crypts or gastric caeca, in the posterior region of the mid-gut that harbour beneficial symbionts belonging to the Gamma- or Beta-Proteobacteria (Buchner 1965, Fukatsu and Hosokawa 2002, Glasgow 1914, Hosokawa *et al* 2010, Kikuchi *et al* 2011, Miyamoto 1961, Prado and Almeida 2009). However, several other symbiotic syndromes occur across Pentatomomorpha, including paired or unpaired bacteriomes with intracellular symbionts in some Lygaeoidea (Kuechler *et al* 2012, Matsuura *et al* 2012), as well as more complex microbial communities in mid-gut regions devoid of crypts (in Pyrrhocoroidea; Sudakaran *et al.* 2012). Thus, evolutionary transitions in symbiotic syndromes must have occurred repeatedly in stinkbugs. Even though such transitions are expected to have

major implications for the functionality of the symbiosis, the evolutionary consequences of changes in symbiotic syndromes remain enigmatic.

Among pentatomomorphan bugs, the Pyrrhocoridae appear to be exceptional with regards to both the localisation of the symbionts and the microbiota composition. Previous studies on *Pyrrhocoris apterus* and *Dysdercus fasciatus* revealed that they harbour a distinct and stable microbiota consisting of obligate and facultative anaerobes such as Actinobacteria (*Coriobacterium glomerans* and *Gordonibacter* sp.), Firmicutes (*Clostridium* sp.) and Gamma-Proteobacteria (*Klebsiella* sp.). These bacteria are localized in the ventricose region (M3) of the midgut (Haas and König 1987, Salem *et al* 2013, Sudakaran *et al* 2012), which is the main region for the digestion of the ingested food particles (Kodrík *et al* 2012, Silva and Terra 1994). Concordantly, the midgut crypts of *P. apterus* and *D. fasciatus* are reduced in size and do not contain any symbiotic microbes (Glasgow 1914, Sudakaran *et al* 2012). Similar crypt morphologies have been reported for other genera such as *Antilochus* and *Probergrothius*, suggesting that the M3-associated microbial community may be widespread among Pyrrhocoridae (Bentz and Kallenborn 1995, Glasgow 1914, Goel and Chatterjee 2003, Rastogi 1964, Singh and Singh 2001). In *P. apterus* and *D. fasciatus*, the gut microbiota was found to be vital for growth and survival of the host (Salem *et al* 2013), through the supplementation of B vitamins by the dual actinobacterial symbionts *C. glomerans* and *Gordonibacter* sp. (Salem *et al* 2014). As the predominant food source of Pyrrhocoridae, i.e. seeds of the plant order Malvales (Kristenová *et al* 2011), is deficient in B vitamins, this symbiont-mediated nutritional upgrading plays an important role by allowing the hosts to exploit a nutritionally inadequate diet (Salem et al. 2013).

In the present study, we aimed to elucidate the ecological and evolutionary implications of a major transition in symbiotic syndromes. Specifically, we tested the hypothesis that

the evolutionary transition to a characteristic mid-gut core microbiota enabled the adaptive radiation of pyrrhocorid bugs on the nutritionally imbalanced diet of Malvales seeds. To this aim, we characterized the microbiota across 25 species of Pyrrhocoroidea (22 Pyrrhocoridae and three Largidae species) by a combination of 454 pyrosequencing and quantitative PCR. Additionally, we reconstructed a dated phylogeny of the hosts through calibration with the fossil record and compared it to a distance dendrogram of microbial community profiles as well as strain-level phylogenies of the two vitamin-provisioning actinobacterial symbionts. The results allow us to assess the distribution of the characteristic M3 midgut microbiota across bugs of the superfamily Pyrrhocoridea and to identify the evolutionary origin of this symbiotic syndrome. Subsequently, a comparison with the age of Malvales plants allows for testing the hypothesis that the acquisition of a specific microbiota preceded the bugs' adaptive radiation on this otherwise deficient food source. Furthermore, host-symbiont co-cladogenetic analyses shed light on the evolutionary stability and maintenance of the characteristic core microbiota in Pyrrhocoridae. Taken together, the results provide novel insights into the evolutionary transitions and ecological relevance of symbiotic microbial communities in the diverse insect order Hemiptera.

4.3 MATERIALS AND METHODS

4.3.1 Insect sample collection and DNA extraction

For characterizing the microbial community, symbiont and host phylogenies across the Pyrrhcoroidea superfamily and outgroup taxa, live adult specimens of Pyrrhocoridae (22), Largidae (3), Lygaeidae (2), Oxycarenidae (1), and Rhopalidae species (1) were collected from their respective habitats across four different continents (Table S1). Bugs were killed and preserved in 70% ethanol until further analysis, and at least one individual per species was kept in ethanol as a voucher specimen. Prior to DNA extraction, the samples were surface-sterilized by rinsing with sterile Millipore water, 1% SDS, and then again sterile Millipore water. Up to six complete specimens per bug species (or fewer, if less than seven individual specimens were available), were homogenized under liquid nitrogen with sterile pestles. For the Japanese bug specimens, however, the dissected mid-gut was used instead of the whole individuals. DNA was extracted using the MasterPure^{TM} DNA Purification Kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. An additional lysozyme incubation step (30 min at 37 °C; 4 µL of 100 mg/mL lysozyme, Sigma-Aldrich, St.Louis, MO, USA) was included prior to proteinase K digestion to break up Gram-positive bacterial cells (see Sudakaran *et al.,* 2012). Individual extracts were used for qPCR analysis, as well as for PCR and sequencing of host and symbiont genes for phylogenetic analysis. Pooled DNA extracts from each species were used for 454 pyrosequencing of the associated bacterial communities.

4.3.2 Reconstruction of the host phylogeny

The phylogenetic relationships among the Pyrrhocoridae, its sister family Largidae and outgroup taxa were reconstructed using PCR amplification and sequencing of two mitochondrial (cytochrome oxidase I and II) and one nuclear gene (18S rRNA) for all host species using primers listed in Table 1. PCR was performed in a total reaction volume of 12.5 μL, containing 1 μL of template DNA, 1×PCR buffer (20 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$ and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 μM dNTPs, 0.8 μM of each primer and 0.5 U of Taq DNA polymerase (VWR International GmbH, Darmstadt, Germany). Cycle parameters were as follows: 3 min at 94° C, followed by 35 cycles of 94° C for 40 s, 55° C for 40 s and 72° C for 40 s, and a final extension step of 4 min at 72° C. PCR products were then sequenced bidirectionally on an ABI 3730xl capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). Protein-coding sequences (COI and COII) were curated and then aligned based on their amino acid translation in Geneious Pro 5.4 (Biomatters, Auckland, New Zealand), while partial 18S rRNA sequences were aligned using the SINA aligner (Pruesse *et al* 2012). The individual alignments were concatenated and used for phylogenetic reconstruction with maximum likelihood algorithms (ML) and Bayesian Inference (BI). An ML tree was computed with FastTree 2.1 using the GTR model, and local support values were estimated with the Shimodaira-Hasegawa test based on 1,000 resamplings without reoptimizing the branch lengths for the resampled alignments (Price *et al* 2010). For BI (computed using MrBayes 3.1.2; Huelsenbeck and Ronquist 2001), the data set was partitioned into the three genes, with six substitution types for the CO genes (GTR model), and one for the ribosomal gene (F81 model). Due to saturation, third codon positions were excluded from the analysis for the two mitochondrial genes (COI and COII). The analysis was performed with four chains and a temperature of 0.2 for 10,000,000 generations, and we confirmed that the

standard deviation of split frequencies was consistently below 0.01. Trees were sampled every 1,000 generations, and a 'burn-in' of 1,000 was used (=10%). We computed a 50% majority rule consensus tree with posterior probabilities for every node.

Table 1: Primers used for phylogenetic reconstruction of Pyrrhocoridae and charaterization of their associated microbiota. Use: (1) general amplication and sequencing of host genes, (2) cloning/sequencing of Coriobacteriaceae symbionts, (3) qPCR, (4) 454 sequencing.

Target	Target organism	Target gene	Primer name	Primer sequence (5´-->3´)	Fwd./Rev.	length	Use	Reference
Hosts	Heteroptera	18S rRNA	Pyr18S_2F	GGGAGGTAGTGACAAAAAATAACG	Fwd.	24	1	Li *et al.* (2005)
	Heteroptera	18S rRNA	Pyr18S_4F	ATCCTTTAACGAGGATCTATTGG	Fwd.	23	1	Li *et al.* (2005)
	Heteroptera	18S rRNA	Pyr18S_3R	ACATACTTGGCAAATGCTTTCGC	Rev.	23	1	Li *et al.* (2005)
	Heteroptera	18S rRNA	Pyr18S_4R	GTTAGAACTAGGGCGGTATCTG	Rev.	22	1	Li *et al.* (2005)
	Heteroptera	COI	C1-J-2183-F	CAACATTTATTTTGATTTTTTGG	Fwd.	23	1	Li *et al.* (2005)
	Heteroptera	COI	TL2-N-3014-R	TCCAATGCACTAATCTGCCATATTA	Rev.	25	1	Li *et al.* (2005)
	Heteroptera	COI	C1-J-2530-F	GGAGTAATTCTAGCCAACTC	Fwd.	20	1	Li *et al.* (2005)
	Heteroptera	COI	C1-N-2609-R	GAATACTGCTCCTATGGATA	Rev.	20	1	Li *et al.* (2005)
	Heteroptera	COII	TK-N3796_rev	ACTATTAGATGGTTTAAGAG	Rev.	18	1	Li *et al.* (2005)
	Heteroptera	COII	TL-J3033_fwd	TCTAATATGGCAGATTAGTGCA	Fwd.	20	1	Li *et al.* (2005)
Symbionts	*Coriobacterium /Gordonibacter*	16S rRNA	Cor-2F	GGTAGCCGGGTTGAGAGACC	Fwd.	20	2	Kaltenpoth *et al.* (2009)
	Eubacteria	16S rRNA	rP2	ACGGCTACCTTGTTACGACTT	Rev.	21	2	Weisburg *et al.* (1991)
	Eubacteria	16S rRNA	M13F	TGTAAAACGACGGCCAGT	Fwd.	18	2	Weisburg *et al.* (1991)
	Eubacteria	16S rRNA	M13R	GGAAACAGCTATGACCATG	Rev.	19	2	Weisburg *et al.* (1991)
	Clostridium	16S rRNA	Clostridium_1050-fwd	CTCGTGTCGTGAGATGTTGG	Fwd.	20	3	Weisburg *et al.* (1991)
	Clostridium	16S rRNA	Clostridium_1248-rev	GCTCCTTTGCTTCCCTTTGT	Rev.	20	3	Weisburg *et al.* (1991)
	Klebsiella	16S rRNA	Klebsiella_250-fwd	CAGCCACACTGGAACTGAGA	Fwd.	20	3	Weisburg *et al.* (1991)
	Klebsiella	16S rRNA	Klebsiella_453-rev	GTTAGCCGGTGCTTCTTCTG	Rev.	20	3	Weisburg *et al.* (1991)
	Gordonibacter	16S rRNA	D.fas_Egg.2R_qPC	CCGTATCTCAGTCCCAATGT	Rev.	20	3	Weisburg *et al.* (1991)
	Gordonibacter	16S rRNA	Act-2F	GCGAACGGGTGAGTAACAC	Fwd.	19	3	Weisburg *et al.* (1991)
	Coriobacterium	16S rRNA	Gray519F	CAGCMGCCGCNGTAANAC	Fwd.	18	3	Weisburg *et al.* (1991)
	Coriobacterium	16S rRNA	Cor-1R	ACCCTCCCMTACCGGACCC	Rev.	19	3	Weisburg *et al.* (1991)
	Eubacteria	16S rRNA	Gray28F	GAGTTTGATCNTGGCTCA	Fwd.	19	4	Weisburg *et al.* (1991)
	Eubacteria	16S rRNA	Gray519R	GTNTTACNGCGGCKGCTG	Rev.	18	4	Weisburg *et al.* (1991)

4.3.3 Dating of the host phylogeny

Divergence time estimations for the Pyrrhocoroidea superfamily were inferred using BEAST v1.8.0 (Drummond and Rambaut 2007) by testing various substitution models and parameter settings (see Table S2 and S3) on a fixed input tree (the BI tree, see above). Two fossil calibration points were used: (i) Two *Dysdercus* fossils from Florissant beds in Colorado (37.0-33.1 mya) (Scudder 1890), and (ii) a *Pyrrhocoris tibialis* fossil from Rott-am-Siebengebirge in Germany (28.5-23.8) (Statz and Wagner 1950). A hard upper boundary for the age of the root was set to 160 ±10 mya, based on to the age of the oldest Pentatomomorpha fossil as well as the estimated age of the Pentatomomorpha infraorder (~152.9 mya, Upper Jurassic) (Li *et al* 2012, Misof *et al* 2014). Evaluation and comparison of model parameters were performed using Tracer

v1.5 (Drummond and Rambaut 2007). The maximum clade credibility (consensus) tree was inferred with TreeAnnotator using a burnin of 5,000 and a posterior probability limit of 0.5 (Drummond and Rambaut 2007). The consensus tree was visualized with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/), including HPD intervals.

4.3.4 Characterization of microbial community profiles

Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) was performed to characterize the microbial community composition of members belonging to Pyrrhocoridae, Largidae and outgroup. DNA was sent to external service providers (Research & Testing Laboratories & MR DNA Lab, TX, USA), and amplification was achieved using the 16S rRNA primers Gray28F and Gray519R (Table 1) (Ishak *et al* 2011, Sun *et al* 2011). Sequencing libraries were generated through one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar high-fidelity *Taq* polymerases (Qiagen, Valencia, CA, USA). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures. All low-quality reads (quality cut-off = 25) and sequences < 200 bp were removed following sequencing, which left between 1,990 and 30,361 sequences per sample for subsequent analysis.

Processing of the high-quality reads was performed using QIIME (Caporaso *et al* 2010b). Sequences were clustered into operational taxonomic units (OTUs) using multiple OTU picking in combination with chimera checking using usearch (Edgar 2010) followed by cdhit (Fu *et al* 2012) with 97% similarity cut-offs. For each OTU, one representative sequence was extracted (the most abundant) and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso *et al* 2010a), with the minimum sequence identity percent set to 75. Taxonomy was assigned using RDP

classifier (Wang *et al* 2007), with a minimum confidence to record an assignment set to 0.80. An OTU table was generated describing the abundance of bacterial phylotypes within each sample (Table S4). The table was then manually curated by removing low-abundance reads (<0.1% in each of the samples) and by blasting the representative sequences (see Supplementary Data S1) against the NCBI and RDP databases. To visualize the results, OTUs with the same genus-level assignments were combined based on the blast results. The genus-level table was used to construct heatmaps using the R package 'gplots (heatmap.2)'. For beta diversity analysis and UPGMA clustering, the raw OTU table was subsampled to the depth of 1,500 sequences per sample, and distance matrices were calculated using Bray-Curtis and Jaccard metrics. For visualization, 2D-PCoA plots and dendrograms based on UPGMA clustering were constructed based on the beta diversity distance matrices.

4.3.5 Quantification of core microbes

Quantitative PCRs (qPCR) were performed for the four dominant bacterial symbionts in Pyrrhocoridae (*Coriobacterium glomerans*, *Gordonibacter* sp., *Clostridium* sp., and *Klebsiella* sp.) using specific 16S rRNA primers (Table 1) on a RotorgeneQ cycler (Qiagen, Hilden, Germany) in final reaction volumes of 25 µL, containing 1 µL of template DNA (usually a 1:10 dilution of the original DNA extract), 2.5 µL of each primer (10 µm), and 12.5 µL of SYBR Green Mix (Rotor-Gene SYBR Green kit, Qiagen). Standard curves were established using 10^{-8}-10^{-2} ng of specific PCR product as templates for the qPCR. A NanoDrop^{TM}1000 spectrophotometer (Peqlab Biotechnology Limited, Erlangen, Germany) was used to measure DNA concentrations for the templates of the standard curve. PCR conditions were as follows: 95° C for 5 min, followed by 35 cycles of 60° C for 30 s, 72° C for 20 s and 95° C for 15 s; then a melting curve analysis

was performed by increasing the temperature from 60° C to 95° C within 20 min. Based on the standard curves, the 16S copy numbers of the four dominant symbionts could be calculated for each individual bug from the qPCR threshold values (Ct) by the absolute quantification method (Lee *et al* 2006, 2008), taking the dilution factor and the absolute volume of DNA extract into account. The absolute 16S copy numbers were log-transformed and then used to visualize the quantitative differences of the bacterial symbionts across different host genera using box plots.

4.3.6 Symbiont (Coriobacteriaceae) strain-level phylogenies

PCR amplifications of the 16S rRNA region of both Coriobacteriaceae symbionts – *Coriobacterium glomerans* and *Gordonibacter* sp. – were carried out with the primers Cor2F and rP2 using a Biometra thermocycler (Analytik Jena, Jena, Germany) in total reaction volumes of 12.5 µL containing 1 µL of template DNA, 1×PCR buffer (20 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$ and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 µM dNTPs, 0.8 µM of each primer and 0.5 U of Taq DNA polymerase (VWR International GmbH, Darmstadt, Germany). Cycle parameters were as follows: 3 min at 94° C, followed by 35 cycles of 94° C for 40 s, 68° C for 40 s, and 72° C for 40 s, and a final extension step of 4 min at 72° C. PCR products were cloned into *E. coli* using the StrataClone PCR Cloning Kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Transformed *E. coli* cells were grown on LB agar containing 10 mg/mL ampicillin and 2% 5-bromo-4-chloro-indolyl-β-d-galactopyranoside (X-gal) (Sigma-Aldrich, St.Louis, MO, USA) for blue/white screening. Colony PCR was performed on eight randomly selected transformants for each insect host with vector primers M13F and M13R (Table 1) using the above-mentioned reaction mix and cycling conditions, except that an annealing temperature of 55 °C was used. PCR products were

checked for the expected size on a 1.5% agarose gel (130 V, 30 min) and purified using the peqGOLD MicroSpin Cycle Pure Kit (Peqlab Biotechnologies GmbH, Erlangen, Germany) prior to sequencing with M13 primers. Nearly full length *Coriobacterium glomerans* and *Gordonibacter* sp. 16S rRNA sequences from different Pyrrhocoridae were obtained by combining the short sequences from OTUs picked for each individual species with bTEFAP and the sequences obtained by PCR/cloning for the respective OTUs. Symbiont 16S rRNA sequences were aligned to reference sequences of Coriobacteriaceae type strains obtained from the Ribosomal Database Project (RDP) (Cole *et al* 2014) using the SINA aligner (Pruesse *et al* 2012). The phylogenetic relationships were computed using ML and BI as described for the host phylogeny.

4.3.7 Cophylogenetic analysis of host and symbiont

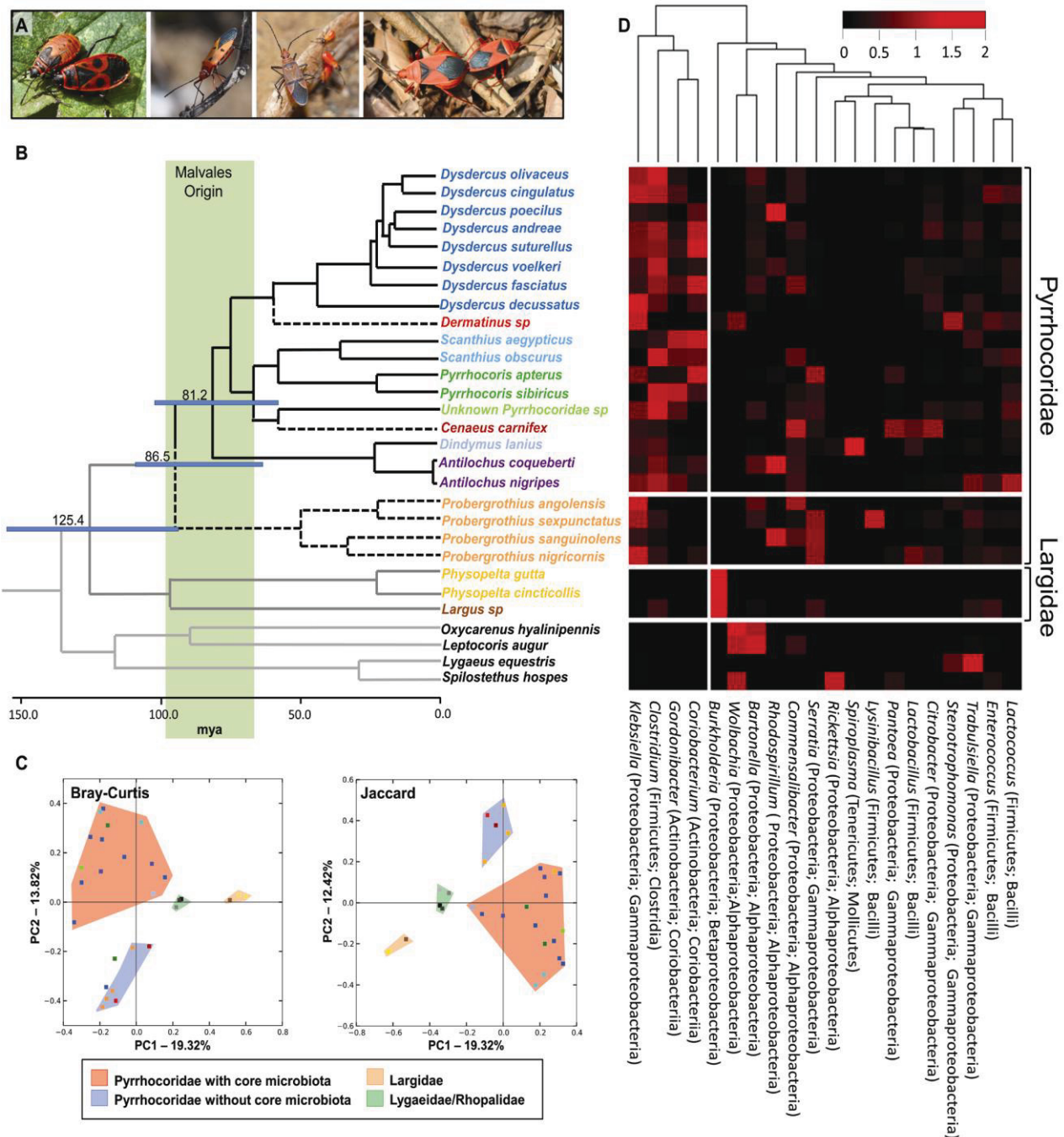
To test for codiversification between hosts and their Coriobacteriaceae symbionts, as well as between the two Coriobacteriaceae symbionts, Treemap 3 (Page 1995) and Parafit (Legendre *et al* 2002) were used. In Treemap, the host and symbiont trees were randomized (1,000 replicates), and the number of observed codiversification events was compared with the resulting distribution of codiversification events in the randomized dataset. For Parafit analysis, host distance matrix was computed using the R package 'Ape (cophenetic.phylo)' based on the phylogenetic tree, and symbiont distance matrices were computed in BioEdit 7.0.5.3 (Hall 1999) based on the concatenated alignments. Permutation tests (1,000 replicates) were run as implemented in ParaFit (Legendre *et al* 2002). In order to assess the possible obscuring effect of interspecific predation on co-phylogenetic patterns, we repeated the analyses after omission of known carnivorous host taxa (*Antilochus* spp., *Dindymus lanius*) that may acquire the Coriobacteriaceae symbionts horizontally via feeding on heterospeficic pyrrhocorid bugs.

4.4 RESULTS

4.4.1 Host phylogeny and divergence time estimates

To elucidate the evolutionary origin of the Pyrrhocoridae-microbiota association, the phylogenetic relationships across bugs of the Pyrrhocoroidea superfamily were reconstructed. The combination of partial 18S rRNA, COI and COII gene sequences resolved most of the taxonomic relationships within the Pyrrhocoroidea (Fig. 1B & S1), and divergence time estimations based on two fossil calibration points and a hard lower boundary for the root age yielded consistent age estimates for selected nodes of interest across a range of different substitution models (GTR+I+G, HKY+G, HKY+I+G, TN93+G, TN93+I+G) and parameter settings (Table S2 and S3). Omitting the *P. tibialis* fossil calibration point did not affect age estimates, while omitting either the *Dysdercus* fossil calibration or the root boundary resulted in significantly increased age estimates

Figure 1. Dated host phylogeny and microbiota profile of 22 species within the family Pyrrhocoridae as well as outgroups (Largidae, Lygaeidae, Oxycarenidae and Rhopalidae). (**A**) Photographs of selected Pyrrhocoridae host species: Adult and fifth instar nymph of *Pyrrhocoris apterus*, adult *Dysdercus cingulatus*, adult and nymphs of *Dysdercus fasciatus,* and a mating pair of *Probergrothius sanguinolens* (from left to right). (**B**) Phylogenetic relationships of the hosts (Pyrrhocoridae n=22, Largidae n=3, Lygaeidae n=2, Oxycarenidae n=1, Rhopalidae n=1 species), reconstructed using partial 18S rRNA, cytochrome oxidase I and cytochrome oxidase II gene sequences. Divergence time estimates were derived using BEAST analyses (TN93+I+G model). Selected node ages are shown in million years ago (mya) with 95% highest posterior density (HPD) interval bars. Dashed branches represent pyrrhocorid taxa without the characteristic core microbiota. The green colored bar indicates the estimated origin of the Malvales (72-96 mya)(Wang *et al* 2009). (**C**) 2D Principal Coordinate Analysis (PCoA) showing the clustering of host species based on their microbial community profiles using Bray-Curtis (left) and Jaccard (right) distance matrices, respectively. Colors for individual samples correspond to the coloring of taxa in Figure 1B. (**D**) Relative abundance of microbial taxa as obtained from 454 pyrosequencing of 16S rRNA amplicons (305,179 reads in total), represented as a heatmap based on log-transformed values. OTUs were combined on the genus level for better visualization (see below heatmap), and only genera that amount to >1% of the microbial community in at least one of the host species are displayed. The dendrogram above the heatmap represents the clustering of microbial taxa according to their distribution and abundance across host species. Note the distinct clustering of the four core microbial taxa associated with Pyrrhocoridae.


A
B
Malvales
Origin
Dysdercus olivaceus
Dysdercus cingulatus
Dysdercus poecilus
Dysdercus andreae
Dysdercus suturellus
Dysdercus voelkeri
Dysdercus fasciatus
Dysdercus decussatus
Dermatinus sp
Scanthius aegypticus
Scanthius obscurus
Pyrrhocoris apterus
Pyrrhocoris sibiricus
Unknown Pyrrhocoridae sp
Cenaeus carnifex
Dindymus lanius
Antilochus coqueberti
Antilochus nigripes
Probergrothius angolensis
Probergrothius sexpunctatus
Probergrothius sanguinolens
Probergrothius nigricornis
Physopelta gutta
Physopelta cincticollis
Largus sp
Oxycarenus hyalinipennis
Leptocoris augur
Lygaeus equestris
Spilostethus hospes
81.2
86.5
125.4
150.0
100.0
50.0
0.0
mya
C
Bray-Curtis
Jaccard
PC2 – 13.82%
PC1 – 19.32%
PC2 – 12.42%
PC1 – 19.32%
Pyrrhocoridae with core microbiota
Pyrrhocoridae without core microbiota
Largidae
Lygaeidae/Rhopalidae
D
0
0.5
1
1.5
2
Pyrrhocoridae
Largidae
Lactococcus (Firmicutes; Bacilli)
Enterococcus (Firmicutes; Bacilli)
Trabulsiella (Proteobacteria; Gammaproteobacteria)
Stenotrophomonas (Proteobacteria; Gammaproteobacteria)
Citrobacter (Proteobacteria; Gammaproteobacteria)
Lactobacillus (Firmicutes; Bacilli)
Pantoea (Proteobacteria; Gammaproteobacteria)
Lysinibacillus (Firmicutes; Bacilli)
Spiroplasma (Tenericutes; Mollicutes)
Rickettsia (Proteobacteria; Alphaproteobacteria)
Bartonella (Proteobacteria; Alphaproteobacteria)
Wolbachia (Proteobacteria;Alphaproteobacteria)
Burkholderia (Proteobacteria; Betaproteobacteria)
Coriobacterium (Actinobacteria; Coriobacteriia)
Gordonibacter (Actinobacteria; Coriobacteriia)
Clostridium (Firmicutes; Clostridia)
Klebsiella (Proteobacteria; Gammaproteobacteria)
Commensalibacter (Proteobacteria; Alphaproteobacteria)
Rhodospirillum (Proteobacteria; Alphaproteobacteria)
Serratia (Proteobacteria; Gammaproteobacteria)

(Table S3). Based on the tracer analysis of effective sample sizes and marginal likelihood values, the TN93+I+G model with two codon partitions for the protein-coding genes (1+2, and 3), estimated base frequencies, and a relaxed uncorrelated lognormal clock model yielded the most robust results.

The phylogenetic analyses revealed an estimated age of 135.7 mya for the superfamily Pyrrhocoroidea (95% highest posterior density [HPD] interval: 104.4–159.9 mya, Fig. 1B). The families Pyrrhocoridae and Largidae formed monophyletic sister taxa that split about 125.4 mya (95% HPD interval: 92.9–154.6 mya, Fig. 1B). Within the Pyrrhocoridae family, the genus *Probergrothius* diverged from the common ancestor of all other taxa about 86.5 mya (95% HPD interval: 63.1–109.3 mya). Around 81.2 mya (95% HPD interval: 58.4–102.2 mya), the clade *Dindymus+Antilochus* split from the group comprising *Dysdercus, Dermatinus, Scanthius, Pyrrhocoris,* the unknown pyrrhocorid taxon, and *Cenaeus*.

4.4.2 Microbial community composition of Pyrrhocoridae bugs

The microbiota of several species of Pyrrhocoridae (n = 22) and Largidae (n = 3), as well as outgroup taxa (n = 4) were characterized using 454 amplicon pyrosequencing of bacterial 16S rRNA (BTEFAP), which yielded a total of 305,179 sequences. The sequences were clustered into 356 OTUs after removing singletons and chimeric sequences as well as OTUs below 0.5% abundance. Bray-Curtis and Jaccard clustering of the host species based on their bacterial community profiles revealed a well-defined cluster containing the genera *Dysdercus*, *Scanthius*, *Pyrrhocoris*, *Dindymus, Antilochus,* and the unknown Pyrrhocoridae species, a second cluster with *Probergrothius*, *Dermatinus* and *Cenaeus*, and separate clusters for members of the Largidae family and the outgroup composed of Pentatomomorphan bugs (*Oxycarenus hyalinipennis*,

Leptocoris augur, *Lygaeus equestris* and *Spilostethus hospes*), respectively (Fig. 1C). UPGMA dendrograms of the bacterial communities associated with the host species computed using the beta diversity matrices (Bray-Curtis & Jaccard) yielded qualitatively similar topologies with the major groupings being identical (Fig. S2).

Combining OTUs on the genus level revealed that the microbiota of Pyrrhocoridae bugs was dominated by four core bacterial taxa: *Coriobacterium glomerans, Gordonibacter* sp. (Actinobacteria), *Clostridium* sp. (Firmicutes), and *Klebsiella* sp. (Proteobacteria) (Fig. 1D). These taxa were almost consistently present across Pyrrhocoridae in abundances ranging from 10^4 to 10^8 16S rRNA gene copies per individual (Fig. 2), with the exception of the genera *Probergrothius*, *Dermatinus,* and *Cenaeus,* which lacked the Coriobacteriaceae symbionts (Fig. 1D and Fig. 2). Furthermore, even though OTUs associated with the genera *Clostridium* and *Klebsiella* were present in most species of these three host genera, qPCR assays specific for the Pyrrhocoridae-associated *Clostridium* and *Klebsiella* OTUs were negative for all samples except two of the *Probergrothius* specimens, indicating that the *Clostridium* and *Klebsiella* OTUs associated with these three genera differ from those of the other Pyrrhocoridae (Fig. 2). Thus, the host genera *Probergrothius*, *Dermatinus,* and *Cenaeus* lacked the microbiota that is characteristic for other Pyrrhocoridae, which is also reflected in their separate clustering in the PCoA analyses (Fig. 1C).

The microbiota of members of the family Largidae (the sister taxon to the Pyrrhocoridae) was dominated by *Burkholderia* and completely lacked the Pyrrhocoridae-associated core microbes (Fig. 1D and 2). Likewise, the core microbiota was absent from other Pentatomomorphan outgroup species: the microbiota of both *Oxycarenus hyalinipennis* (Oxycarenidae) and *Leptocoris augur* (Rhopalidae) was dominated by *Wolbachia* and *Bartonella*, while the Lygaeidae species *Lygaeus equestris* and *Spilostethus hospes*

contained consortia of *Trabulsiella* and *Stenotrophomonas (L. equestris),* or *Wolbachia* and *Rickettsia (S. hospes),* respectively.

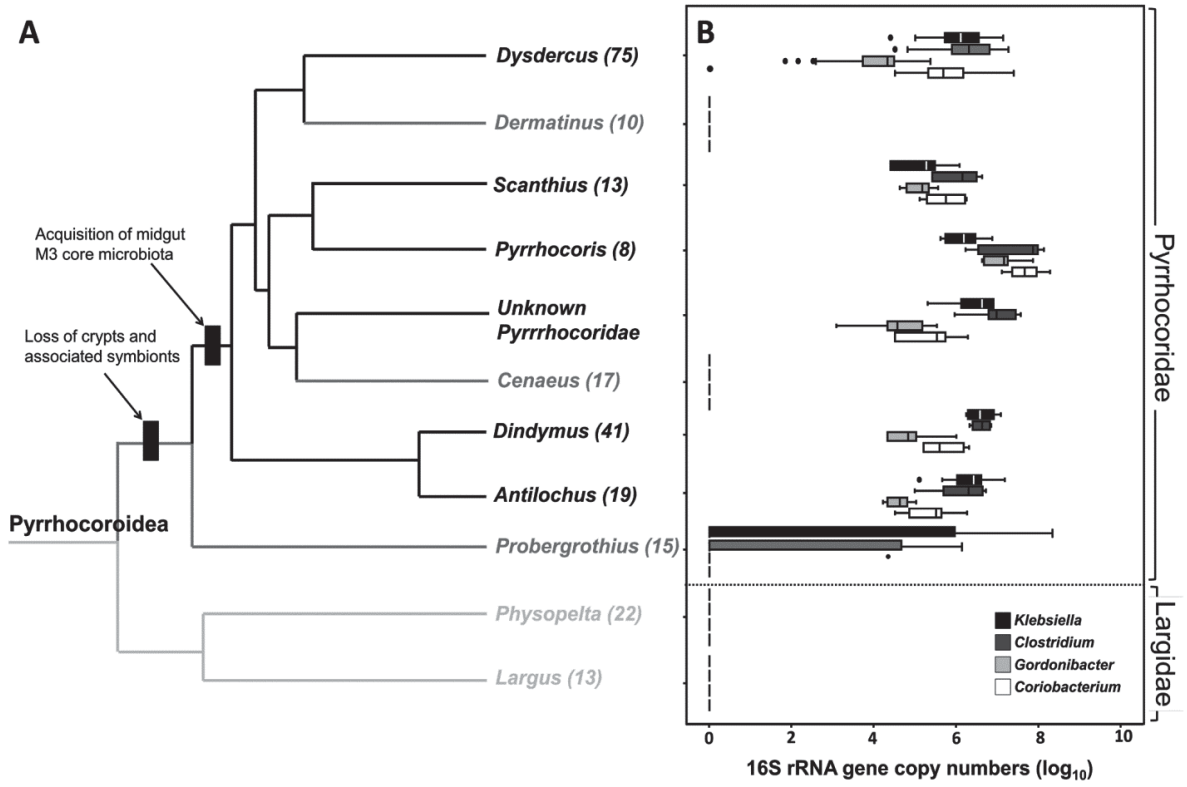


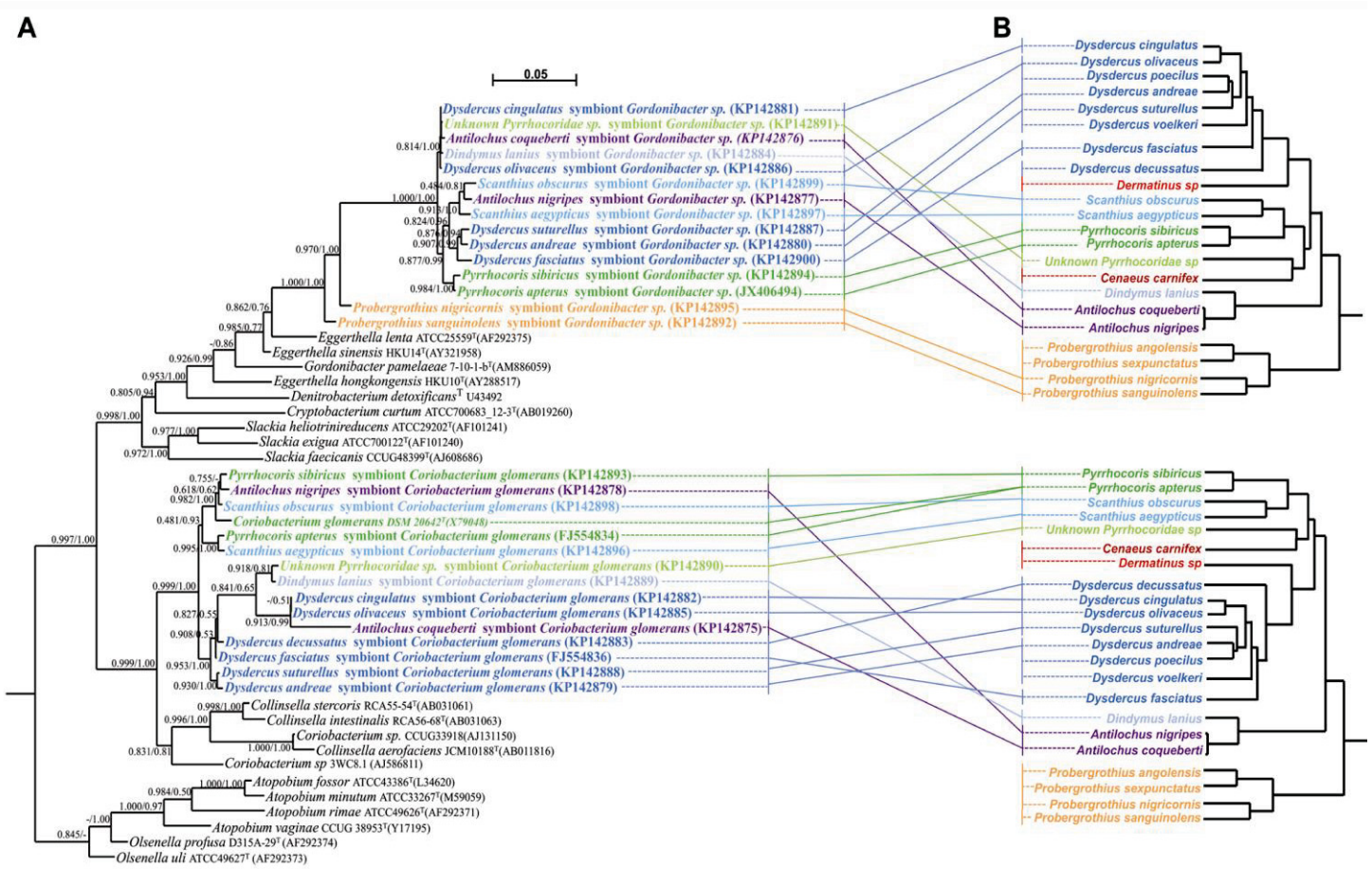
Figure 2. Evolutionary transitions in symbiotic syndromes in Pyrrhocoroidea, and abundance of core microbial taxa. (**A**) Schematic phylogeny of Pyrrhocoridae and Largidae genera (adapted from Fig. 1B). Pyrrhocoridae taxa with core microbiota are given in black, those taxa without the core microbiota are in dark grey, and the Largidae with crypt-associated symbionts are in light grey. Numbers of validly described extant species are given behind each genus name (from (Hussey 1929) (**B**) Abundance of the four core symbiont taxa (*Coriobacterium glomerans*, *Gordonibacter* sp., *Clostridium* sp. and *Klebsiella* sp.) across multiple specimens of the nine different genera of Pyrrhocoridae [*Dysdercus* (n = 37), *Dermatinus* (n = 2), *Scanthius* (n = 5), *Pyrrhocoris* (n = 7), *Unknown Pyrrhocoridae* (n = 6), *Cenaeus* (n = 5), *Dindymus* (n = 6), *Antilochus* (n = 7) and *Probergrothius* (n = 23)] and two genera of Largidae [*Physopelta* (n = 4) and *Largidae* (n = 1)]. Abundance was assessed as 16S rRNA gene copy numbers using qPCR, based on replicate individuals per host species, which were then combined on the genus level. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range.

4.4.3 Phylogenetic analysis of the Coriobacteriaceae symbionts

The Pyrrhocoridae core microbiota contains two actinobacterial symbionts that were previously shown to be essential for growth and survival in *P. apterus* and *D. fasciatus* through the supplementation of B vitamins (Salem *et al* 2013, 2014). We reconstructed the phylogeny of both Coriobacteriaceae symbionts based on the successful amplification and sequencing of the symbionts' 16S rRNA gene sequences from 13 different host species, as well as only *Coriobacterium glomerans* from *Dysdercus decussatus,* and only *Gordonibacter* sp. from two *Probergrothius* species (i.e. *P. nigricornis* and *P. sanguinolens*). The phylogenetic analyses revealed that the symbiotic *Coriobacterium glomerans* and *Gordonibacter* sp. strains form two distinct monophyletic clades within the family Coriobacteriacae, which is consistent with a single acquisition event for each symbiont and subsequent host-symbiont coevolution (Fig. 3). Within each clade, however, the phylogenies of both Coriobacteriaceae symbionts are incongruent with the Pyrrhocoridae host phylogeny (*Coriobacterium glomerans*: Parafit: P = 0.974, TreeMap: P = 0.345; *Gordonibacter* sp.: Parafit: P = 0.978, TreeMap: P = 0.449) (Fig. 3). To exclude the possibility that co-phylogenetic patterns were obscured by interspecific predation among pyrrhocorid bugs resulting in transient Coriobacteriaceae being picked up in the bTEFAP sequences, we repeated the analyses after excluding known predatory taxa (*Antilochus* spp., *Dindymus lanius*). Despite some clustering of symbiont taxa according to their host genera (particularly *Pyrrhocoris* and *Scanthius* for *Gordonibacter*, and *Dysdercus* for *Coriobacterium*), the tests for co-cladogenesis remained non-significant. Thus, even though the Pyrrhocoridae maintain a specific microbiota, horizontal transmission between co-occurring species apparently played an important role during the evolution of the symbiosis. Since the Coriobacteriaceae symbionts are both localized in the same region of the mid-gut and can be co-transmitted both vertically and

horizontally (Kaltenpoth *et al.* 2009), we also tested for co-cladogenesis of the two symbiont lineages. Randomization of phylogenetic trees or distance matrices and subsequent statistical evaluation, however, yielded no evidence for co-cladogenesis between *Coriobacterium glomerans* and *Gordonibacter* sp. strains across host taxa (Parafit: $P = 0.898$, TreeMap: $P = 0.251$).

Figure 3. Co-phylogenetic analysis of **(A)** the dual actinobacterial symbionts (*Coriobacterium glomerans* and *Gordonibacter* sp.) and **(B)** their Pyrrhocoridae hosts**.** The symbiont phylogeny is based on partial 16S rRNA sequences. Colors for individual symbiont strains correspond to the coloring of host taxa in Figure 1B. Host-symbiont associations are shown by connecting lines. Values at the nodes represent local support values from the FastTree analysis (GTR model) and Bayesian posterior probabilities, respectively.



4.5 DISCUSSION

In this study, we characterized the microbiota associated with bugs of the Hemipteran families Pyrrhocoridae and Largidae, and we investigated the origin and evolutionary dynamics of the host-microbiota association on community and strain levels. The results yield insights into an evolutionary transition from individual crypt-associated symbionts to a more complex microbiota localized in the mid-gut. This transition coincided with the evolution of the hosts' preferred food plants and preceded the major radiation of pyrrhocorid bugs, highlighting the importance of the microbial community in adapting to novel ecological niches.

4.5.1 Nutritional contributions of the core microbiota associated with pyrrhocorid host

Members of the Pyrrhocoridae family are predominantly phytophagous, feeding on seeds of the plant order Malvales, with a few notable exceptions such as *Probergrothius angolensis*, which feeds on seeds of the ancient gymnosperm *Welwitschia mirabilis* (Wetschnig and Depisch 1999). Despite being phylogenetically distant, these host plants share similar phytochemical defenses, particularly cylcopropenoic fatty acids (CPFAs) (Allen *et al* 1967). These compounds are known to be toxic to insects, due to the inhibition of fatty acid desaturation (Allen *et al* 1967). Interestingly, the noxious effects of CPFAs are particularly problematic under B vitamin starvation conditions, as artificial supplementation of vitamins partly abolished the adverse effects of CPFAs in rats (Schneider *et al* 1968). Since the seeds of Malvales plants are known to be deficient in B vitamins (Whitsitt 1933), the combined effect of vitamin deficiency and CPFAs likely poses severe nutritional challenges to insects attempting to exploit this food source.

In previous studies, we have shown that *P. apterus* and *D. fasciatus*, two members of the Pyrrhocoridae family, harbor a stable and specific mid-gut microbiota dominated by four microbial taxa: two Actinobacteria (*Coriobacterium glomerans*, *Gordonibacter* sp.), one Firmicute (*Clostridium* sp.), and one Proteobacterium (*Klebsiella* sp.), irrespective of the geographical origin or diet of the bugs (Salem *et al* 2013, Sudakaran *et al* 2012). Our present characterization of the microbiota associated with 22 different species belonging to nine genera of Pyrrhocoridae using 16S rRNA amplicon pyrosequencing and quantitative PCR revealed the presence of the same dominant bacterial taxa across six (*Dysdercus*, *Scanthius*, *Pyrrhocoris*, *Dindymus, Antilochus,* and the unidentified specimen) out of the nine investigated genera of Pyrrhocoridae, but absent from the other three genera as well as all of the non-Pyrrhocoridae outgroup taxa, including the sister family Largidae (Fig. 1 and 2).

The presence of a consistent core microbiota across most Pyrrhocoridae suggests an important role of the symbionts for host fitness. Concordantly, experimental removal of the actinobacterial symbionts by egg surface sterilization had a strong negative effect on the fitness of the host, with severely reduced survival during development and strongly reduced fecundity of adult individuals (Salem *et al* 2013). Furthermore, a recent study combining fitness assays of Actinobacteria-deprived and control bugs on an artificial diet with transcriptome sequencing of the host and genome analysis of one of the Coriobacteriaceae symbionts (*C. glomerans*) revealed that these symbionts supplement the nutrition of the host with limiting B vitamins (Salem *et al* 2014), which may also mitigate the toxic effects of the plants' CPFAs in the bugs' gut (Schneider *et al* 1968). Thus, the Coriobacteriaceae symbionts are tightly integrated into the hosts' metabolism and play an important role for nutrient provisioning and, possibly, detoxification (Salem et al. 2014). The third core microbial taxon, *Clostridium* sp., belongs to the

Lachnospiracae (Firmicutes), whose members are anaerobic fermenters. Their production of butyric acid can reduce the abundance of bacterial pathogens in the insect gut by stimulating mucin and antimicrobial peptide production (Hamer *et al* 2008) and/or serve as a nutrient source to the host (Meehan and Beiko 2014). Given that many *Clostridium* species have cellulolytic capabilities (Lynd *et al* 2002), the symbiont could also contribute to cellulose digestion. However, as Lachnospiraceae have not yet been described as symbionts of insects other than Pyrrhocoridae, their contribution to host fitness remains speculative. The fourth symbiont, *Klebsiella* sp., belongs to the Enterobacteriaceae (Gamma-Proteobacteria). *Klebsiella* are facultative anaerobes associated with diverse eukaryotic organisms as commensals or opportunistic pathogens (Bagley 1985, Podschun and Ullmann 1998). Some strains are capable of fixing atmospheric nitrogen to be utilized by plants (Cakmakci *et al* 1981) as well as insects (Leaf-cutter ants; Pinto-Tomas *et al* 2009). These symbiont-provided benefits may enable their insect hosts to successfully exploit a nutritionally inadequate food source (the seeds of Malvales plants) that is unpalatable to many other insects, due to the low concentrations of available B vitamins, and the presence of toxic CPFAs.

4.5.2 Evolutionary origin of the Pyrrhocoridae-microbiota association

The M3 core microbiota of Pyrrhocoridae is distinctly different from that of their closest relatives, the Largidae, which harbored more or less a monoculture of *Burkholderia* (Fig. 1D). Related *Burkholderia* symbionts have been described from the mid-gut crypts of several other bug taxa in the superfamilies Coreoidea and Lygaeoidea (Kikuchi *et al* 2011). As Largidae also possess well-defined mid-gut crypts (Glasgow 1914), a crypt localization seems very likely for their *Burkholderia* symbionts. Given the absence of

Burkholderia or any other dominant symbiont taxon (Fig. 1D) as well as the simple structure of the mid-gut crypts in the basal pyrrhocorid genus *Probergrothius* (Goel and Chatterjee 2003, Rastogi 1964), the Pyrrhocoridae likely lost crypt-associated symbionts soon after the evolutionary split from the Largidae, which occurred around 125.2 mya ago (Fig. 1A, Fig. 2). The core microbiota consisting of *Coriobacterium glomerans, Gordonibacter* sp.*, Clostridium* sp.*,* and *Klebsiella* sp., however, was not established before the split of *Probergrothius* from the rest of the pyrrhocorids in the late Cretaceous (81.2-86.5 mya) (Fig. 1 and Fig. 2). Thus, the pyrrhocorid symbiosis with its extracellular core microbiota is significantly younger than most of the known intracellular nutritional mutualisms in insects, such as the aphid–*Buchnera* (160–280 mya; Moran *et al* 1993), cockroach–*Blattabacterium* (135–250 mya; Bandi *et al* 1995), planthopper–*Vidania* (>130 mya; Urban and Cryan 2012), and Auchenorrhyncha–*Sulcia* symbioses (260–280 mya; Moran *et al* 2005). Importantly, however, the estimated age of the Pyrrhocoridae core microbiota coincides with the inferred origin of their host plant order Malvales (72-96 mya, (Wang *et al* 2009)) (Fig. 1). Even though host and symbiont contributions towards exploitation of the diet cannot be completely disentangled at present, the increased diversity in pyrrhocorid bugs after the acquisition of the M3-localized anaerobic core microbiota suggests an important contribution of the symbionts towards the adaptive radiation of their bug hosts to utilize Malvales seeds as a nutritional resource (Fig. 2).

Our results indicate that some pyrrhocorid genera apparently lack the characteristic microbiota. As mentioned above, *Probergrothius* likely diverged from the rest of the Pyrrhocoridae before the acquisition of the core microbiota. Even though this genus shows the occurrence of *Clostridium sp.*, the symbiont differs from the taxonomically related OTUs of the other pyrrhocorids, and the two actinobacterial symbionts are entirely

lacking in *Probergrothius*. Despite the absence of these symbionts, some of the *Probergrothius* species successfully exploit Malvales seeds, while others feed on *Welwitschia* seeds (Goel and Chatterjee 2003, Wetschnig and Depisch 1999), which contain CPFAs as well (Aitzetmuller and Vosmann 1998). How these bugs fulfill their dietary requirements of B vitamins and prevent the toxic effects of CPFAs is currently unknown. In this context, however, it is noteworthy that one of our Lygaeidae outgroup taxa, *Oxycarenus hyalinipennis*, co-exists in the same environment as several other Pyrrhocoridae and also feeds on Malvales seeds (Saxena and Bhatnagar 1958). Despite sharing the same ecological niche, *O. hyalinipennis* harbors a completely different microbiota comprising mainly *Wolbachia* sp. and *Bartonella* sp., indicating that different adaptations have evolved independently to cope with the nutritional challenges associated with feeding on Malvales seeds.

In contrast to the primary lack of the core microbiota in *Probergrothius* spp., *Cenaeus carnifex* and *Dermatinus* sp. lost the core microbiota secondarily (Fig. 1B). Although the Pyrrhocoridae predominantly feed on Malvales seeds, some species including members of the genera *Cenaeus, Pyrrhocoris, Antilochus,* and *Dindymus* have been reported to predominantly (*Antilochus* and *Dindymus*) or occasionally (*Cenaeus* and *Pyrrhocoris*) utilize dead insects or other animals as food source (Ahmad and Schaefer 1987, Socha 1993). A carnivorous supplementation of an otherwise phytophagous diet could provide essential nutrients (including B vitamins) and thereby relax the selective pressures to maintain a nutrient-supplementing microbial community. This scenario could explain the loss of symbionts in *Cenaeus* and possibly *Dermatinus*. In this context, however, it is interesting to note that even though the genera *Antilochus* and *Dindymus* have been characterized as predominantly carnivorous (Ahmad and Schaefer 1987, Ari Noriega and Huay Lee 2010, Jackson and Barrion 2002, Kohno *et al* 2004), they retained the core

microbiota in qualitatively and quantitatively similar composition as other Pyrrhocoridae genera (Fig. 1 and 2). Although it is possible that the core bacteria in *Antilochus* and *Dindymus* represent transient associates acquired via feeding on sympatrically occurring pyrrhocorid bugs, their numerical abundance implies a contribution to host fitness. Thus, the link between the carnivorous supplementation of the predominantly phytophagous diet and the secondary loss of symbionts in some Pyrrhocoridae requires further investigation, which should particularly focus on the functional role the gut-associated microbial communities play in different Pyrrhocoridae genera.

4.5.3 Mixed transmission mode and partner specificity

In insect-microbe interactions, functionally relevant and vertically transmitted microbial symbionts are expected to co-evolve with their host, resulting in the congruence of host and symbiont phylogenies. Such patterns have been well-documented in several insects harboring primary intracellular endosymbionts (Moran *et al* 2008). More recently, changes in microbial community profiles have also been suggested to mirror the evolutionary relationships among host taxa in symbiotic associations that remain stable over long evolutionary timescales (Brucker and Bordenstein 2012). In the present study, the gut microbiota of the Pyrrhocoridae family remains both quantitatively and qualitatively stable across most host species. However, there are multiple discrepancies between the host phylogeny and the symbiont relationships on the community and strain level (*C. glomerans* and *Gordonibacter* sp.), with overall no statistical support for co-cladogenesis, strongly implying horizontal transmission of symbionts between heterospecific hosts (Fig. 3). It is well documented in *P. apterus* that even though the symbionts are predominantly transmitted vertically from mother to offspring through egg smearing, symbiont-deprived bugs in the laboratory readily acquire the microbes

horizontally from conspecifics through coprophagy (Kaltenpoth *et al* 2009). A second possible explanation for the horizontal exchange of symbionts is the tendency among some Pyrrhocoridae bugs to occasionally predate on other co-occurring pyrrhocorid species. Similar findings of a predominantly vertical transmission with a low rate of horizontal transmission (i.e. mixed transmission mode) in a range of other insects challenge the traditional view of a strict vertical transmission of most insect mutualists and instead highlight the importance of mixed transmission modes across a wide range of host taxa (Ebert 2013). Examples for this include burying beetles (Kaltenpoth and Steiger 2014), fruit flies (Aharon *et al* 2013), beewolves (Kaltenpoth *et al* 2014), termites (Schauer *et al* 2012), and bees (Koch and Schmid-Hempel 2011).

While the combination of vertical and horizontal transmission seems beneficial for the bacterial symbionts, it may be problematic for the host, as selection is expected to favor increased virulence in horizontally transmitted symbionts (Sachs and Wilcox 2006). In such a situation, the host should evolve mechanisms to prevent the establishment of non-native symbionts and/or sanction non-cooperative individuals (Sachs *et al* 2011). Concordantly, three independent observations provide preliminary evidence for partner choice in the Pyrrhocoridae symbiosis: (i) The phylogenies of the two Coriobacteriaceae symbionts (*Coriobacterium glomerans* and *Gordonibacter* sp.) that co-occur consistently across Pyrrhocoridae exhibit no significant pattern of co-cladogenesis. Since both symbionts are expected to be co-transmitted vertically (via egg smearing) as well as horizontally (via coprophagy), phylogenetic incongruence suggests differential success of establishment after horizontal transmission events, which may be due to host control. (ii) Antimicrobial peptides such as c-type lysozyme and pyrrhocoricin were found to be up-regulated in the midgut of symbiotic vs. aposymbiotic *D. fasciatus,* suggesting a specific immune response towards symbiont infection that may prevent the invasion of

opportunistic bacteria (Bauer *et al* 2014). (iii) High mortality was observed after the experimental exchange of the core microbiota between heterospecific Pyrrhocoridae individuals (Salem *et al* 2013), indicating that a high degree of specificity characterizes the Pyrrhocoridae symbiosis and is required to maintain a mutualistic partnership.

4.5.4 Major transitions in symbiotic syndromes in the hemipteran infraorder Pentatomomorpha

Symbiotic associations with bacteria have been extensively studied in the hemipteran infraorder Pentatomomorpha, because the diversity of symbiont taxa, localizations, and transmission routes provides interesting insights into evolutionary transitions in symbiotic syndromes. Many Pentatomomorpha possess specialized crypts in the posterior midgut M4 region that harbor primary symbionts (Glasgow 1914) (Fig. 4). Given the occurrence of mid-gut crypts and associated symbionts across four of the five Pentatomomorphan superfamilies, they likely represent the ancestral localization of symbionts in the Pentatomomorpha excluding the fungivorous Aradoidea (Fig. 4). In the superfamily Pentatomoidea, most crypt-associated symbionts are vertically transmitted γ-Proteobacteria that contribute to the nutrition of their hosts (Abe *et al* 1995, Hosokawa *et al* 2005, Kaiwa *et al* 2010, Kaiwa *et al* 2014, Kikuchi *et al* 2009, Prado *et al* 2006) (Fig. 4). By contrast, bugs belonging to the Coreoidea superfamily and several families within the Lygaeoidea harbor *Burkholderia* symbionts in their mid-gut crypts. In some cases, such as in the bean bug *Riptortus pedestris,* every host generation acquires these symbionts *de novo* from the environment (Kikuchi *et al* 2007) (Fig. 4). Several other families in the Lygaeoidea have secondarily lost the crypt-inhabiting symbionts, and some evolved bacteriomes that house a distinct clade of γ-Proteobacteria (Kuechler *et al* 2012, Matsuura *et al* 2012) (Fig. 4).

Based on the occurrence of crypt-associated *Burkholderia* symbionts across Coreoidea and Lygaeoidea, previous studies have assumed the *Burkholderia* symbiosis originated in the ancestor of these two sister taxa (Fig. 4) (Kikuchi et al. 2011a). However, our findings of highly abundant *Burkholderia* in all three investigated Largidae species, together with previous reports on complex and well defined mid-gut crypts in this family (Glasgow 1914, Miyamoto 1961), indicate that the association with *Burkholderia* is more ancient than previously thought. It originated most likely after the split of the Pentatomoidea from the clade comprising the Pyrrhocoroidea, Coreoidea, and Lygaeoidea (Fig. 4). Under this scenario, *Burkholderia* symbionts localized in mid-gut crypts also constituted the ancestral case for the Pyrrhocoroidea, with a subsequent loss of crypts and *Burkholderia* in the Pyrrhocoridae. Interestingly, even though seed feeding is widespread among pentatomomorphan bugs, the transition to an anaerobic microbiota in the midgut lumen appears to be confined to the Pyrrhocoridae. As discussed above, the specialized nutritional challenges associated with the switch to the Malvales host plants may have driven this evolutionary transition in symbiotic syndromes.

Evolutionary transitions in symbiotic associations have been described repeatedly across different insect taxa (Bennett and Moran 2013, Hansen and Moran 2014, Koga *et al* 2013). Evidently, extracellularly localized and transmitted symbionts are more flexible than intracellular associations, because they provide more opportunities to replace less beneficial symbionts. Nevertheless, even replacements of obligate intracellular symbionts have been described in several Auchenorrhynchan lineages (Bennett and Moran 2013, Koga *et al* 2013, Koga and Moran 2014), as well as in aphids (Koga *et al* 2003) and weevils (Toju *et al* 2013). In the Pyrrhocoridae, the symbiotic association transitioned from specialized crypt-associated symbionts to a specific mid-gut localized bacterial community. Several transitions between extracellular symbionts harbored in crypts or in

the lumen of the midgut and intracellular symbionts housed in bacteriomes have been observed in related insect families within the Hemiptera (Bennett and Moran 2013, Hansen and Moran 2014, Kikuchi *et al* 2011, Kuechler *et al* 2012, Matsuura *et al* 2012), but the ecological and evolutionary implications of transitions in symbiotic syndromes remain little understood. This study provides insights into the importance of changes in symbiont localization and identity for the invasion of a novel ecological niche and the subsequent adaptive radiation of the insect host. Future studies on the functional relevance of microbial symbionts during such transitions in symbiotic syndromes might reveal general principles in the ecological factors and evolutionary constraints that underlie niche expansions and adaptive radiations.

Host taxon		Symbiotic Localization	Bacterial Symbiont		References
Superfamily	Families		Phylum	Genus/Species	
Lygaeoidea	Blissidae; Geocoridae; Lygaeidae; and Oxycarenidae	Paired and unpaired bacteriomes attached to different parts of ovaries or midgut	γ-Proteobacteria	'*Candidatus* Schneideria nysicola'; '*Candidatus* Ischnodemia utricula'; '*Candidatus* Arocatia carayoni'; '*Candidatus* Kleidoceria schneideri'	Kuechler *et al* 2012; Matsuura *et al* 2012
	Berytidae; Blissidae; Pachygronthidae; and Rhyparochromidae	Midgut crypts	β-Proteobacteria	*Burkholderia sp.*	Kikuchi *et al* 2011a
	Malcidae	---	---	---	Kikuchi *et al* 2011a
Coreoidea	Alydidae; Coreidae; and Stenocephalidae	Midgut crypts	β-Proteobacteria	*Burkholderia sp.*	Kikuchi *et al* 2011a
	Rhopalidae	---	---	---	Kikuchi *et al* 2011a
Pyrrhocoroidea	Pyrrhocoridae	Midgut M3 region	Actinobacteria, Firmicutes, and γ-Proteobacteria	*Coriobacterium glomerans*; *Gordonibacter sp.*; *Clostridium sp.*; *Klebsiella sp.*	This study
	Largidae	Midgut crypts	β-Proteobacteria	*Burkholderia sp.*	This study
Pentatomoidea	Acanthosomatidae; Cydnidae; Parastrachiidae; Pentatomidae; Plataspidae; Scutelleridae; And Urostylidae	Midgut crypts	γ-Proteobacteria	'*Candidatus* Rosenkranzia clausaccus'; '*Candidatus* Ishikawaella capsulata'; '*Candidatus* Benitsuchiphilus tojoi'; *Erwinia sp.*	Hosokawa *et al* 2005, 2012; Kikuchi *et al* 2009; Kaiwa *et al* 2010, 2014; Prado *et al* 2006
Aradoidea	Aradidae	---	---	---	

Figure 4. Symbiotic syndromes (i.e. symbiont identity and localization) in bug families of the infraorder Pentatomomorpha. The superfamily-level phylogenetic relationships of the hosts were obtained from earlier studies based on the 18S rRNA (Xie *et al* 2005) and whole mitochondrial genomes (Hua *et al* 2008). The grey bar indicates the previously hypothesized evolutionary origin of crypt-associated *Burkholderia* in stinkbugs (Kikuchi *et al* 2011), while the black bar indicates the revised origin of this association, based on the discovery of *Burkholderia* symbionts in Largidae (this study).

4.6 CONCLUSIONS

This study provides the first comprehensive characterization of the microbiota associated with different species of Pyrrhocoridae and yields comprehensive insights into the evolution of this mutualistic symbiosis. The evolution of the specific core microbiota in Pyrrhocoridae during the late Cretaceous coincided with the origin of their preferred host plants (Malvales), suggesting that the symbionts were instrumental in allowing the host to exploit and diversify in this ecological niche. Pyrrhocorid bugs represent one of the few established and experimentally amenable systems that can be used to address fundamental questions on the interactions of multiple bacterial symbionts within the gut of an insect host. The exploration of the symbionts' functional importance and the molecular basis of host-symbiont interactions may shed new light on the role microbial partners played for the evolutionary success of insects.

4.7 ACKNOWLEDGMENTS

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4.9 SUPPLEMENT

Table S1: Collection localities and Genbank accession numbers of Pyrrhocoridae, Largidae, Oxycarenidae, Rhopalidae and Lygaeidae used for the characterization of the microbiota, host phylogeny and symbiont phylogeny.

			Number of	NCBI accession number		
Species	**Family**	**Collected in**	**specimens**	**18s**	**COI**	**COII**
Dysdercus olivaceus	Pyrrhocoridae	India	6	KP142856	KP142924	
Dysdercus cingulatus	Pyrrhocoridae	India	6	KP142853	KP142923	
Dysdercus poecilus	Pyrrhocoridae	Japan	1	KP142857	KP142928	
Dysdercus andreae	Pyrrhocoridae	USA	5	KP142852	KP142926	
Dysdercus suturellus	Pyrrhocoridae	USA	5	KP142858	KP142925	
Dysdercus voelkeri	Pyrrhocoridae	Côte d'Ivoire	6	KP142859	KP142916	
Dysdercus fasciatus	Pyrrhocoridae	Côte d'Ivoire	6	KP142855	KP142927	
Dysdercus decussatus	Pyrrhocoridae	Japan	2	KP142854	KP142918	
Dermatinus sp.	Pyrrhocoridae	India	2	KP142849	KP142939	
Scanthius aegypticus	Pyrrhocoridae	USA	2	KP142873	KP142943	
Scanthius obscurus	Pyrrhocoridae	India	3	KP142872	KP142922	KP142915
Pyrrhocoris apterus	Pyrrhocoridae	Germany	6	KP142870	KP142932	KP142914
Pyrrhocoris sibiricus	Pyrrhocoridae	Japan	1	KP142871	KP142931	
Unknown Pyrrhocoridae	Pyrrhocoridae	India	6	KP142851	KP142930	KP142905
Cenaeus carnifex	Pyrrhocoridae	South Africa	5	KP142848	KP142929	KP142903
Dindymus lanius	Pyrrhocoridae	India	6	KP142850	KP142919	KP142904
Antilochus coqueberti	Pyrrhocoridae	Japan	1	KP142846		KP142901
Antilochus nigripes	Pyrrhocoridae	India	6	KP142847	KP142920	KP142902
Probergrothius angolensis	Pyrrhocoridae	Namibia	6	KP142869	KP142942	KP142913
Probergrothius sexpunctatus	Pyrrhocoridae	Côte d'Ivoire	5	KP142868	KP142921	
Probergrothius sanguinolens	Pyrrhocoridae	India	6	KP142867	KP142938	KP142912
Probergrothius nigricornis	Pyrrhocoridae	India	6	KP142866	KP142917	KP142911
Physopelta gutta	Largidae	Japan	2	KP142865	KP142941	KP142910
Physopelta cincticollis	Largidae	Japan	2	KP142864	KP142940	KP142909
Largus sp.	Largidae	USA	1	KP142860	KP142937	KP142906
Oxycarenus hyalinipennis	Oxycarenidae	India	6	KP142863	KP142935	KP142908
Leptocoris augur	Rhopalidae	India	6	KP142861	KP142936	KP142907
Lygaeus equestris	Lygaeidae	Russia	6	KP142862	KP142933	
Spilostethus hospes	Lygaeidae	India	6	KP142874	KP142934	

Table S2: Model parameters of the phylogenetic dating analyses using BEAUti and BEAST. For each analysis, 25 million steps were performed with tree sampling every 2,500 steps. A burnin of 5,000 and a posterior probability limit of 0.5 were used for tree reconstruction. The best model according to Tracer analysis (see Table S3) is highlighted in bold print.

Analysis	Gene	Codon	Substitution	Base			Age priors		
number	part.	part.	model	frequencies	Clock model	Starting tree	Root	*Dysdercus* fossils	*Pyrrhocoris* fossil
1	3	(1+2, 3)	GTR+I+G	estimated	strict	user-specified (ML), fixed	Uniform: 160 - 0	lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
2	3	(1+2, 3)	GTR+I+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed	Uniform: 160 - 0	lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
3	3	(1+2, 3)	HKY+I+G	estimated	strict	user-specified (ML), fixed	Uniform: 160 - 0	lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
4	3	(1+2, 3)	HKY+I+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed	Uniform: 160 - 0	lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
5	3	(1+2, 3)	HKY+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed	Uniform: 160 - 0	lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
6	3	(1+2, 3)	TN93+I+G	estimated	strict	user-specified (ML), fixed	Uniform: 160 - 0	lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
7	**3**	**(1+2, 3)**	**TN93+I+G**	**estimated**	**relaxed uncorrlogn**	**user-specified (ML), fixed**	**Uniform: 160 - 0**	**lognormal: 34.1 + 0.5, off: 20**	**lognormal: 25 + 0.5, off: 12**
8	3	(1+2, 3)	TN93+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed	Uniform: 160 - 0	lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
9	3	(1+2, 3)	GTR+I+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed		lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
10	3	(1+2, 3)	TN93+I+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed		lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
11	3	(1+2, 3)	HKY+I+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed		lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
12	3	(1+2, 3)	HKY+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed		lognormal: 34.1 + 0.5, off: 20	lognormal: 25 + 0.5, off: 12
13	3	(1+2, 3)	HKY+I+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed		lognormal: 34.1 + 0.5, off: 20	
14	3	(1+2, 3)	HKY+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed		lognormal: 34.1 + 0.5, off: 20	
15	3	(1+2, 3)	HKY+I+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed			lognormal: 25 + 0.5, off: 12
16	3	(1+2, 3)	HKY+G	estimated	relaxed uncorrlogn	user-specified (ML), fixed			lognormal: 25 + 0.5, off: 12

Table S3: Results of the phylogenetic dating analyses using BEAUti and BEAST. Model parameters for the respective analyses are given in Table S2. The best model according to Tracer analysis is highlighted in bold print.

			Resulting age estimates (mya)								
Analysis	Tracer analysis		Pyrrhocoridae - Largidae split			Probergrothius split from Pyrrhocoridae			Min. age of Pyrrhocoridae w/out Probergrothius		
number	ESS	marg. likelihood	median	lower HPD	upper HPD	median	lower HPD	upper HPD	median	lower HPD	upper HPD
1	*bad*	-14526.86	132.1	107.8	154.3	87.2	70.0	102.3	82.8	66.5	97.5
2	*bad*	-14509.64	126.9	95.4	155.7	86.7	63.4	109.2	81.2	58.0	101.4
3	*good*	-14565.79	131.0	105.7	154.2	88.1	70.1	104.3	83.5	66.2	98.6
4	*bad*	-14550.79	125.7	93.7	155.4	86.8	62.0	109.3	81.2	58.3	103.4
5	*good*	-14703.14	125.2	92.3	154.4	87.1	62.9	110.5	81.7	58.6	103.8
6	*good*	-14586.47	130.9	105.5	154.0	87.3	69.7	103.4	83.0	66.5	98.7
7	***good***	**-14570.63**	**125.4**	**92.9**	**154.6**	**86.5**	**63.1**	**109.3**	**81.2**	**58.4**	**102.2**
8	*good*	-14673.12	123.6	90.0	154.6	87.2	62.5	111.6	81.8	57.9	104.3
9	*bad*	-14508.65	171.1	90.2	263.8	114.3	64.2	176.1	106.9	58.8	163.3
10	*good*	-14571.47	165.1	90.6	253.6	111.3	61.6	167.8	104.3	60.4	159.9
11	*good*	-14550.53	164.6	91.0	254.8	111.4	62.9	170.3	104.2	58.7	159.2
12	*good*	-14703.11	161.2	88.9	248.0	110.2	62.2	166.2	103.2	58.9	156.5
13	*good*	-14550.57	123.2	65.6	196.6	83.4	46.3	131.9	78.1	42.3	122.1
14	*good*	-14702.64	121.6	63.8	192.8	83.0	45.5	131.0	77.8	42.5	122.4
15	*bad*	-14591.54	217.0	78.2	393.3	146.8	55.0	264.9	137.6	52.8	250.3
16	*good*	-14702.44	212.0	77.2	383.0	144.4	52.3	258.2	135.3	49.6	243.9

Figure S1. Phylogenetic relationships of Pyrrhocoroidea bugs (Pyrrhocoridae n=22, Largidae n=3, Lygaeidae n=2, Oxycarenidae n=1, Rhopalidae n=1 species), reconstructed using partial 18S rRNA, cytochrome oxidase I and cytochrome oxidase II gene sequences. Values at the nodes of the host phylogeny represent local support values from the FastTree analysis (GTR model), and Bayesian posterior probabilities, respectively.

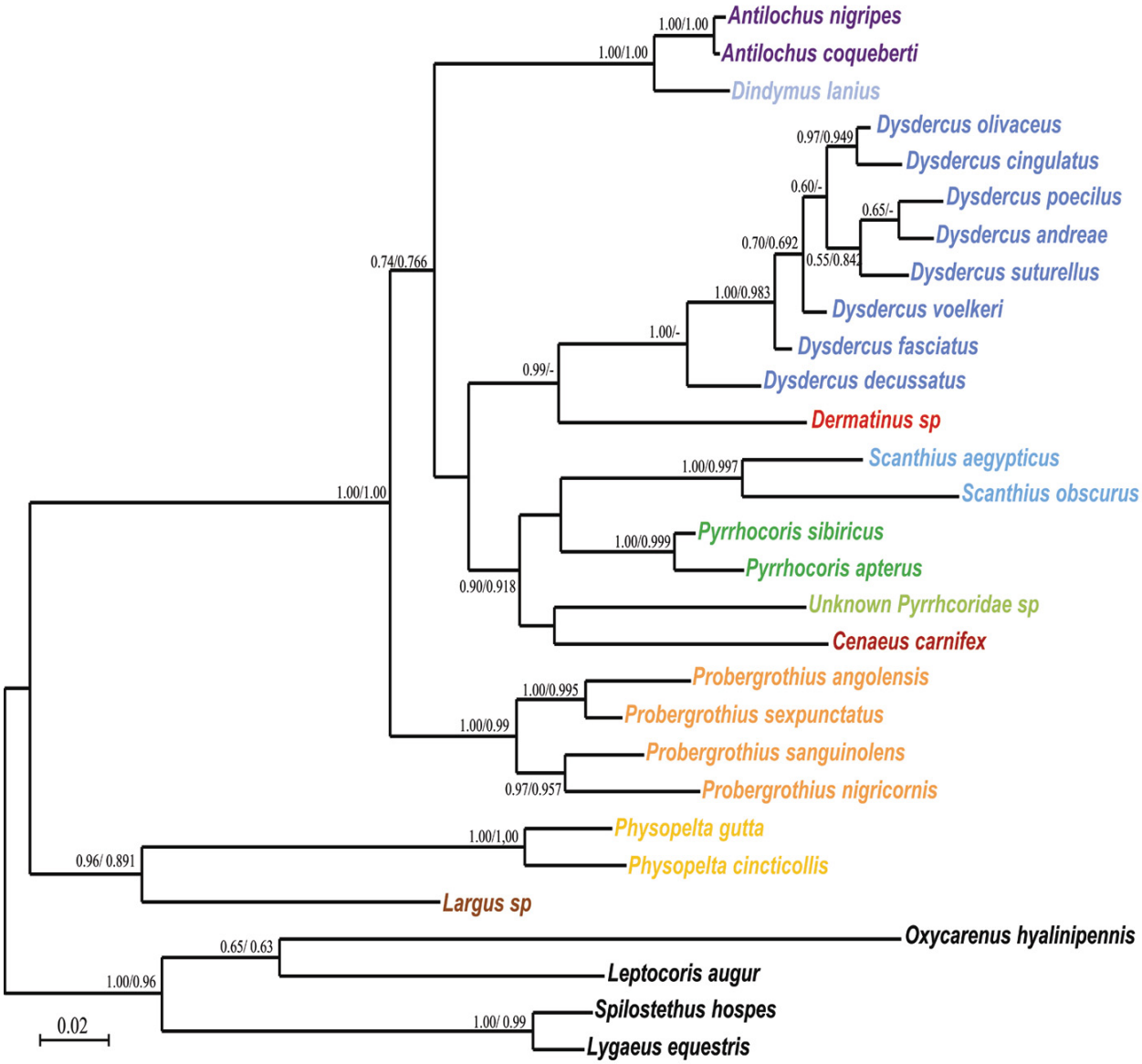
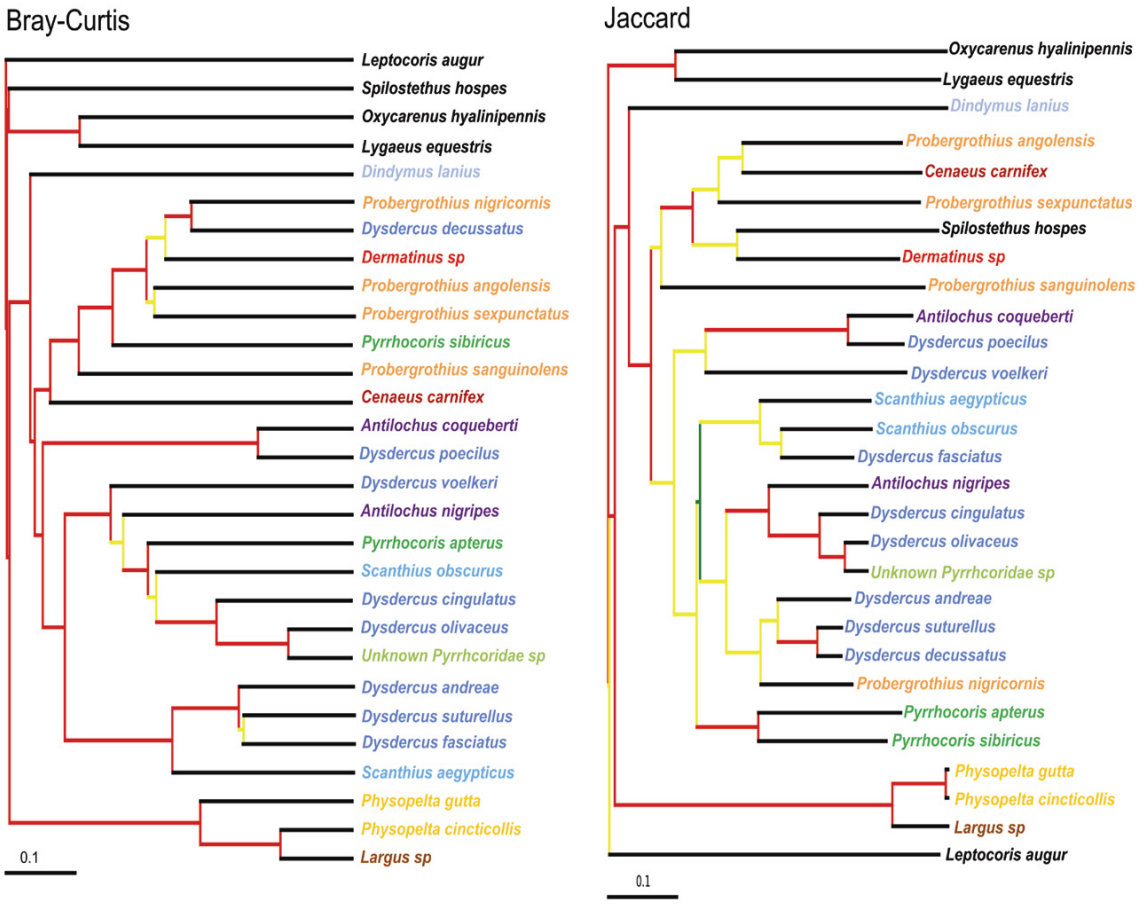


Figure S2. UPGMA clustering of bacterial community profiles across the 22 species within the family Pyrrhocoridae as well as outgroups (Largidae, Lygaeidae, Oxycarenidae and Rhopalidae), based on Bray-Curtis (left) and Jaccard (right) distance matrices, respectively. Colors of internal nodes in the dendrogram represent support values (red – 75-100%, yellow – 50-75%, green – 25-50%, blue <25%). Coloring of host taxa correspond to the coloring in Figure 1B.



CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5

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Symbiont-mediated host plant adaptation in the megadiverse insect order Hemiptera

5.1 PLANT-INSECT INTERACTION INFLUENCED BY MICROBES

Symbiotic associations with microbes are ubiquitous in nature and represent major driving forces of evolutionary innovation by conferring novel phenotypic traits to the host and thereby allowing for the expansion into novel ecological niches and subsequent lineage diversification (Janson *et al* 2008, Schluter 2000, Takiya *et al* 2006). A prime example is the acquisition of aerobic heterotrophic bacteria by a proto-eukaryote that later evolved into mitochondria, which enabled the diversification of eukaryotes from strictly anaerobic protists into the three major multicellular kingdoms (Baldauf 2003). Similarly, mycorrhizal fungi's association with the majority of plant lineages has often been viewed as a prerequisite for their transition from aquatic to terrestrial habitats, supported by the ability of the fungi to contribute essential nutrients to the plants. This hypothesis is supported by the evolution of this relationship coinciding with the age of the oldest known fossil record of land plants (~400 mya) (Newman and Reddell 1987, Simon *et al* 1993).

Plants and insects together constitute for the majority of diversity in terrestrial ecosystems. Evolutionary adaptation to herbivory on angiosperms during cretaceous period is one of the major milestones that contributed to the rich species diversity observed in insects (Jermy 1984, Strong *et al* 1984). The plant-insect interaction is an endless arms race in which plants are continuously evolving to avoid herbivory attacks. Their tissues are composed of complex plant polymers such as cellulose, hemicellulose, pectin and lignin that are difficult or impossible to digest for herbivores (Watanabe and Tokuda 2010). Along with the low nitrogen levels and imbalanced amino acid profiles that can severely hamper the growth of the insects (Mattson 1980, Sandstrom and Moran 1999). Finally the plants also harbor a wide range of toxic allelochemicals to protect against herbivore attacks. Consequently, the insects had to evolve morphological, metabolic and behavioural adaptation to overcome the plant defenses and nutritional challenges (Farrell and Mitter 1994).

In parallel to the adaptive changes in the insects themselves, their association with microbial symbionts can have a profound influence on the interaction with their host plants (Douglas 2009). Symbiotic microbes possess the ability to supplement limiting nutrients, break down fastidious plant polymers, and detoxify plant allelochemicals, thereby directly impacting the insect's ability to exploit a certain host plant as a nutritional resource (Douglas 2009, Janson *et al* 2008, Moran 2007). While a plethora of studies have elucidated the nutritional contributions of microbial symbionts to herbivorous insects (Douglas 2009, Hansen and Moran 2014), we are only beginning to understand their impact on the hosts' diversification following the expansion into novel ecological niches. In this review, we explore the evolutionary implications of transitions in symbiotic associations for dietary switches and adaptive radiations, using the well-studied megadiverse insect order Hemiptera as a model.

5.2 DIVERSITY OF SYMBIOTIC SYNDROMES IN INSECTS

For the purpose of this review, we define a "symbiotic syndrome" to encompass the identity of a microbial symbiont as well as its localization inside the insect host. A diverse range of symbiotic syndromes with nutritional benefits occurs across insect taxa. First, many obligate symbionts are localized intracellularly in specialized organs called bacteriomes with in the insect body cavity. These symbionts are transmitted vertically between generations within the eggs and usually supplement limiting nutrients to their insect host that are deficient in their plant diet (Baumann 2005a). The second category comprises facultative symbionts that infect their host sporadically and can be either intracellular or extracellular, with various routes of vertical and horizontal transmission. These symbionts are mainly known for their ability to manipulate the reproductive capacity of their hosts, but on some occasions can also provide benefits such as defense and nutrition (Feldhaar 2011). The third group is extracellular microbes that infect the insect guts and are localized in the gut lumen or in gut-associated sacs (caeca). These symbionts are often transmitted vertically through egg surface contamination, coprophagy, or special symbiont containing capsules or mucus, but can also be horizontally acquired in every generation from the environmental or from conspecifics by social transmission (Kikuchi *et al* 2011a, Salem *et al* 2014, Sudakaran *et al* 2012) (Salem et al., in press 2015). Gut symbionts can assist in breaking down plant polymers, nutrient provisioning, nitrogen recycling, and detoxification of plant defenses (Brune 2014). Finally, some microbial symbionts live outside of the host's body on the food source, assisting in its break-down to simple substrates suitable for consumption or enriching the diet with assimilated nitrogen. Such interactions are common in the gardens or galleries of fungus-farming insects (Aylward *et al* 2012, Pinto-Tomas *et al* 2009).

Each symbiotic syndrome has its unique functional capabilities constrained by the symbionts' identity and localization, as well as the evolutionary stability of their association with the host insect. For example, intracellular symbionts that form long-term mutualistic associations with their host insects contribute important nutritional benefits, but are limited in their ability to secrete products into the oral secretion or the gut lumen to break down complex compounds or detoxify plant toxins, as they are confined inside the cytoplasm of the host cell (Moran *et al* 2008). Conversely, gut symbionts or environmental food-associated microbes are well-suited for such functions (Brune 2014). Furthermore, intracellular symbionts undergo massive gene loss over evolutionary timescales, and horizontal acquisition of new genes is severely restricted, limiting the symbionts metabolic flexibility (McCutcheon and Moran 2012). If a phytophagous insect shifts from its current host plant to a nutritionally different resource, its intracellular symbionts are unlikely to compensate the novel nutritional challenges. As a result, the insect host is forced to either replace the current symbiont with a better suited one or acquire a co-primary mutualist to overcome the new nutritional challenges. By contrast, extracellular gut symbionts or food associated microbes often possess dynamic genomes featuring the ability to gain and lose functional genes, as is typical for most bacterial taxa. However, their transmission between generations might be less reliable, making their contributions towards host fitness evolutionarily less stable. In both scenarios, shifts in symbiotic syndromes can be expected to be selected for in insect lineages that exploit novel host plants to compensate for the nutritional and defensive limitations.

5.3 ECOLOGICAL DIVERSITY IN THE INSECT ORDER HEMIPTERA

The order Hemiptera represents the most diverse among the hemimetabolous insect groups, and hemipteran insects radiated into a wide range of ecological niches. The order consists of around 82,000 described species in four suborders, i.e. Auchenorrhyncha, Coleorrhyncha, Sternorrhyncha and Heteroptera (Cryan and Urban 2012). While the Sternorrhyncha (aphids, whiteflies and scale insects), Auchenorrhyncha (cicadas, spittlebugs and planthoppers) and Coleorrhyncha (mossbugs) primarily feed on the nutritionally imbalanced diet of phloem and xylem saps (Redak *et al* 2004, Sandstrom and Moran 1999), the Heteroptera (true bugs) exhibit more diverse food preferences, including the reproductive parts of plants like the flower, ovules, and seeds, as well as in some cases carnivorous diets and even vertebrate blood (Schaefer and Panizzi 2000). As a result of the predominantly phytophagous diet, many Hemiptera are important agricultural pests, and some of the blood feeders are vectors of human diseases. Most hemipteran taxa are associated with symbiotic microbes (Fig. 1) that complement the deficiencies of the food source (Buchner 1965). However the role of transitions in symbiotic syndromes in mediating the adaptive radiation of their host insect lineages into novel ecological niches remains poorly understood.

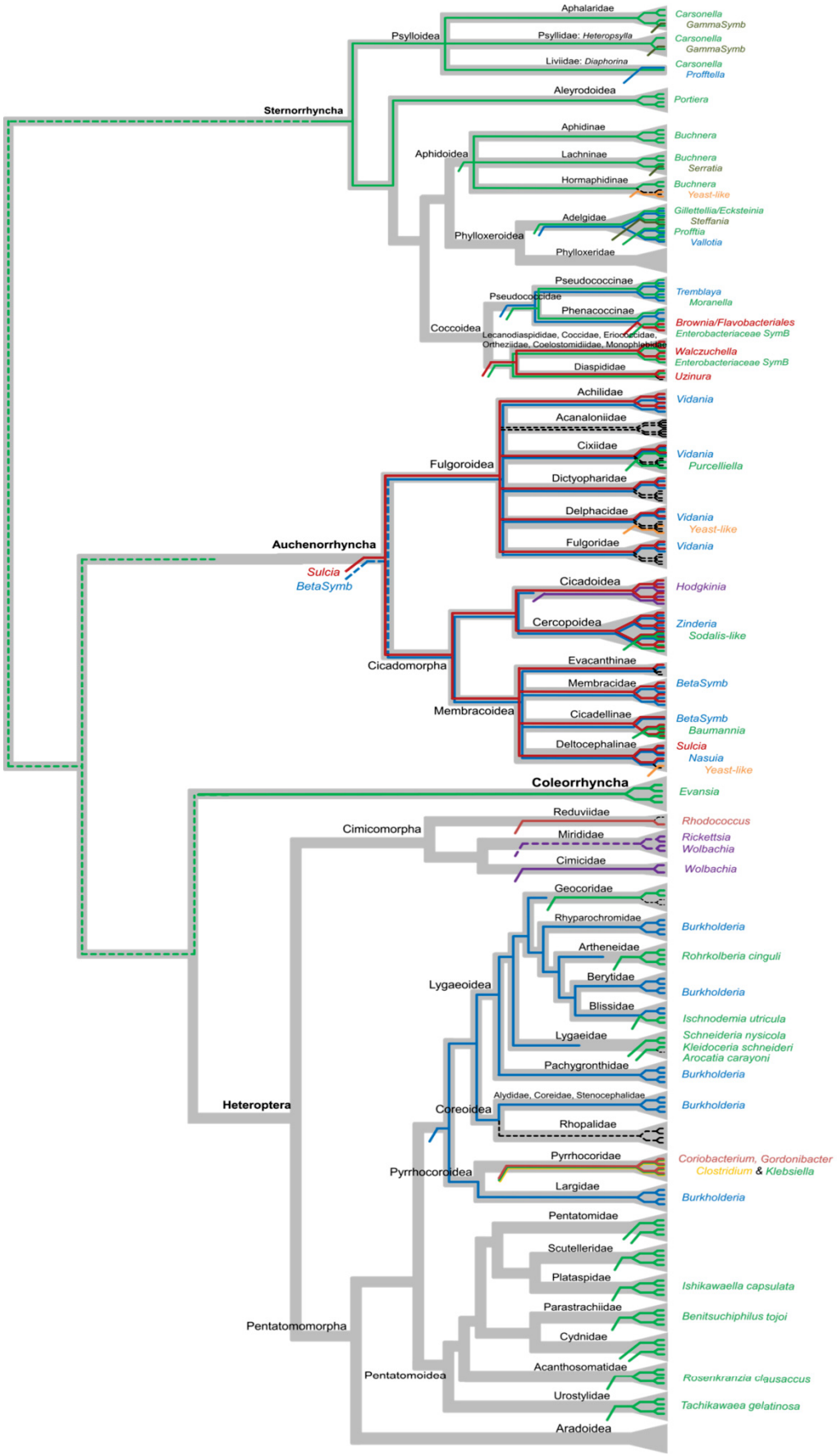
Aphalaridae
Carsonella
GammaSymb
Psylloidea
Psyllidae: Heteropsylla
Carsonella
GammaSymb
Liviidae: Diaphorina
Carsonella
Profftella
Aleyrodoidea
Portiera
Sternorrhyncha
Aphidinae
Buchnera
Aphidoidea
Lachninae
Buchnera
Serratia
Hormaphidinae
Buchnera
Yeast-like
Adelgidae
Gillettellia/Ecksteinia
Steffania
Profftia
Vallotia
Phylloxeroidea
Phylloxeridae
Pseudococcinae
Tremblaya
Moranella
Pseudococcidae
Phenacoccinae
Brownia/Flavobacteriales
Enterobacteriaceae SymB
Coccoidea
Lecanodiaspididae, Coccidae, Eriococcidae,
Orthezziidae, Coelostomidiidae, Monophlebidae
Walczuchella
Enterobacteriaceae SymB
Diaspididae
Uzinura
Achilidae
Vidania
Acanaloniidae
Cixiidae
Vidania
Purcelliella
Fulgoroidea
Dictyopharidae
Delphacidae
Vidania
Yeast-like
Auchenorrhyncha
Fulgoridae
Vidania
Sulcia
BetaSymb
Cicadoidea
Hodgkinia
Cercopoidea
Zinderia
Sodalis-like
Cicadomorpha
Evacanthinae
Membracidae
BetaSymb
Membracoidea
Cicadellinae
BetaSymb
Baumannia
Deltocephalinae
Sulcia
Nasuia
Yeast-like
Coleorrhyncha
Evansia
Reduviidae
Rhodococcus
Cimicomorpha
Miridae
Rickettsia
Wolbachia
Cimicidae
Wolbachia
Geocoridae
Rhyparochromidae
Burkholderia
Artheneidae
Rohrkolberia cinguli
Berytidae
Lygaeoidea
Burkholderia
Blissidae
Ischnodemia utricula
Lygaeidae
Schneideria nysicola
Kleidoceria schneideri
Arocatia carayoni
Pachygronthidae
Burkholderia
Heteroptera
Alydidae, Coreidae, Stenocephalidae
Coreoidea
Burkholderia
Rhopalidae
Pyrrhocoridae
Coriobacterium, Gordonibacter
Clostridium & Klebsiella
Pyrrhocoroidea
Largidae
Burkholderia
Pentatomidae
Scutelleridae
Plataspidae
Ishikawaella capsulata
Parastrachiidae
Benitsuchiphilus tojoi
Pentatomomorpha
Cydnidae
Acanthosomatidae
Rosenkranzia clausaccus
Pentatomoidea
Urostylidae
Tachikawaea gelatinosa
Aradoidea

Figure 1: Schematic summary of the evolutionary transition of symbiotic syndromes across the hemipteran order. The transitions events of the microbial symbionts within the hemipteran order is based on microbial symbiont and insect molecular phylogenetic studies (Bennett and Moran 2013, Cryan and Urban 2012, Grazia *et al* 2008, Hansen and Moran 2014, Kuechler *et al* 2012, Matsuura *et al* 2012, Sudakaran *et al*., submitted). The host insect phylogeny is indicated in grey. The symbionts are color coded based on their taxonomical identity at class level: Gammaproteobacteria – Green, Betaproteobacteria – blue, Alpha proteobacteria – purple, Firmicutes – red, Actinobacteria – Brown and yeast like symbionts – Orange. When gammaproteobacterial symbionts were either replaced with or coexist with another gammaproteobacterial symbiont then the new partner is indicated in darker green. The figure encompasses the long term association maintained between several groups and their bacterial symbionts within Hemiptera and also on the other hand the plasticity of the insect systems in acquiring and losing with a relative frequency to adapt and exploit novel ecological niches.

5.4 SYMBIONT-ASSISTED PHLOEM FEEDING IN STERNORRHYNCHA

In the suborder Sternorrhyncha, most taxa belonging to the four superfamilies Aphidoidea (aphids), Aleyrodoidea (whiteflies), Coccoidea (Pseudococcidae; mealybugs) and Psylloidea (psyllids) are primarily feeding on phloem sap that is rich in sugars but poor in amino acids, vitamins, and co-factors (Sandstrom and Moran 1999). By contrast, armored scale insects (Coccoidea: Diaspididae) and woolly conifer aphids (Phylloxeroidea: Adelgidae) have shifted to feeding on the mesophyll and parenchyma tissue (Sabree *et al* 2013, Toenshoff *et al* 2012). The genomic and experimental analysis of symbionts associated with sternorrhynchan insects revealed the microbial provisioning of essential amino acids as well as cofactors and other beneficial compounds to their respective hosts. These symbiont-provided benefits enabled the exploitation of phloem sap as a primary food source and allowed for the radiation of Sternorrhyncha on a diverse range of host plants. Interestingly, despite their functional relevance, sternorrhynchan symbionts have experienced multiple replacements and transitions from individual to dual symbioses in the course of their evolutionary history (Fig. 1). However, the key metabolic contribution of microbial symbionts across sternorrhynchan symbiotic syndromes remains the

provisioning of the host with essential amino acids, even though additional supplementation with cofactors or involvement in nitrogen recycling occasionally occurs.

'*Candidatus* Carsonella ruddii' and '*Candidatus* Portiera aleyrodidarum' (both Gammaproteobacteria), the intracellular symbionts of psyllids (Psylloidea) and whiteflies (Aleyrodoidea), respectively, are closely related to each other (Baumann 2005b). It is possible that the symbiont colonization occurred in their shared ancestor, and the symbionts subsequently diversified along with their insect hosts. *Carsonella* is harbored in specialized bacteriomes located in the abdominal region of the host. It has one of the smallest genomes (160 kb) identified so far (Nakabachi *et al* 2006) that has also lost pathways responsible for the biosynthesis of some essential amino acids needed by the host (Sloan and Moran 2012b). Some species of psyllids have compensated by acquiring a second obligate intracellular symbiont that inhabits the syncytial cells between the bacteriocytes within the bacteriome (Subandiyah *et al* 2000), and provides the nutritional supplements for which the biosynthetic capabilities were lost in *Carsonella* (Sloan and Moran 2012b). In addition to metabolically complementing *Carsonella* to supplement essential amino acids to the host, '*Candidatus* Profftella armatura' (Betaproteobacteria), a co-primary symbiont of *Carsonella ruddii* in the Asian citrus psyllid *Diaphorina citri,* also encodes a 70-kb locus (15% of it genome) for the production of a defensive toxin (Nakabachi *et al* 2013). However, some other taxa such as *Pachypsylla* spp. and *Heteropsylla texana* are not associated with additional symbionts to compensate for the essential amino acid deficiency (Sloan and Moran 2012b). One plausible explanation for their reduced dependence on the symbiont-provided amino acids is that these psyllids modify their host plant physiology in a way that provides sufficient amounts of several essential amino acids for which the biosynthetic capabilities were subsequently lost in *Carsonella*. Concordantly, the hackberry psyllids of genus *Pachypsylla* elicit dramatic

changes in the host plant during the formation of galls (Price *et al* 1987), and *H. texana* – although not a gall former – also causes extensive morphological alterations to the leaf and floral shoot structures of its *Prosopis spp.* host plants (Donnelly 2002). A second possible explanation is that the host has horizontally acquired genes for the biosynthesis of some essential amino acids from the symbiont or other bacterial associates. Even though the functional relevance of horizontally acquired genes has rarely been substantiated in eukaryotic hosts, several events of symbiont to host gene transfer have been reported in insects (Dunning Hotopp *et al* 2007, Husnik *et al* 2013).

In whiteflies, *Portiera* provisions the host with essential amino acids as well as carotenoids, which are limiting in phloem sap (Sloan and Moran 2012a). Other phloem-feeding insects have independently evolved a different solution for acquiring their carotenoids through the horizontal acquisition of a fungal gene and its integration into the insect genome (Moran and Jarvik 2010). Interestingly, *Portiera aleyrodidarum* of the whitefly species *Bemisia tabaci* is the only known obligate intracellular symbiont to have undergone genome rearrangements and expansion (Sloan and Moran 2013), which may play a role for the evolutionary success of the *Portiera* symbiont and its widespread presence and persistence across whiteflies.

The symbiont of most aphids (Aphidoidea), '*Candidatus* Buchnera aphidicola' (Gammaproteobacteria), provides essential amino acids and riboflavin to the host and also assists in nitrogen recycling (Hansen and Moran 2011, Shigenobu *et al* 2000). Even though *Buchnera* is associated with the majority of aphid species, it was replaced by yeast-like extracellular symbionts in the clade comprising the aphid genera *Cerataphis*, *Tuberaphis*, *Hamiltonaphis* and *Glyphinaphis* (Fukatsu and Ishikawa 1996). In cedar aphids (*Cinara cedri), Buchnera* coexists with the additional intracellular symbiont '*Candidatus* Serratia symbiotica' (Lamelas *et al* 2011). Interestingly, the genome of

Buchnera in *C. cedri* (425 kb) is one of the smallest of all *Buchnera* genomes described so far, having lost several functional genes important to fulfill the nutritional needs of the aphid, with the respective functions being provided by the coexisting *Serratia* symbiont (Lamelas *et al* 2011). *Serratia symbiotica* has been previously identified in other aphid species (*Acrythosiphon pisum*) as a facultative symbiont involved in defense against environmental heat stress (Burke *et al* 2010). A comparative analysis of *Serratia* genomes indicates that they are in the initial stages of genome reduction and transitioning from facultative to obligate endosymbiotic lifestyle in cedar aphids (Lamelas *et al* 2011).

The Adelgidae (woolly conifer aphids) belong to the superfamily Phylloxeroidea, a sister clade to aphids (Aphidoidea). Adelgids are known to feed on parenchyma cells instead of phloem sap in pine, spruce and other conifers, and ancestrally harbor two phylogenetically different bacteriocyte-associated symbionts belonging to Betaproteobacteria and Gammaproteobacteria, respectively (Toenshoff *et al* 2012). Two events of symbiont replacement have been reported within the Adelgidae: First, the ancestral gammaproteobacterial symbiont '*Candidatus* Gillettellia colleyia'/'*Candidatus* Ecksteinia adelgidicola' of *A. cooleyi/A. voweni* and *A. nordmannianae/A. piceae,* respectively, was replaced by the gammaprotebacterial symbiont lineage comprising '*Candidatus* Proffita tarda' and '*Candidatus* Proffita virida' in the ancestor of *Adelges laricis/A. tardus* and *Adelges abietis/A. viridis,* respectively (Toenshoff *et al* 2012). And second, the betaproteobacterial symbiont '*Candidatus* Vallotia' was replaced in the *Adelges nordmannianae/A. piceae* complex by the Gammaproteobacterium '*Candidatus* Steffania'. These transitions of the symbionts in adelgids could be related to the change in the nutritional demands of the insect host due to the shift to a novel food source (parenchyma cells) and subsequent switches in host trees (Toenshoff *et al* 2012). Based on genomic data for '*Ca.* Steffania adelgidicola', all the genes responsible for the

biosynthesis of key metabolites (Essential amino acids, vitamins and co factors) are still intact, highlighting an important nutritional role for the host (Donnelly 2002). In the adelgids' sister family Phylloxeridae, no primary symbiont has been identified so far. Further genomic and experimental data are required to get a better understanding of the symbionts' functional role in aphids of the superfamily Phylloxeroidea.

In the last sternorrhynchan superfamily Coccoidea, several transitions in symbiotic syndromes have been observed (von Dohlen *et al* 2001). Mealybugs (Pseudococcidae) are associated with a unique nested symbiosis composed of '*Candidatus* Tremblaya princeps' (Betaproteobacteria) and its intracellular bacterial symbiont '*Candidatus* Moranella endobia' (Gammaproteobacteria)(von Dohlen *et al* 2001). The two symbionts metabolically complement each and provision essential amino acids to their host (McCutcheon and von Dohlen 2011). Interestingly, one particular species of Pseudococcidae, *Phenacoccus avenae*, harbors only *Tremblaya phenacola* and lacks the *Moranella* symbiont. However, this *Tremblaya* symbiont retains a larger gene set encoding a full set of essential amino acids required by the host, likely representing a more ancestral state of metabolic capabilities in *Tremblaya* (Husnik *et al* 2013). Both *Tremblaya* and *Moranella* have been replaced by a symbiotic assemblage of a Flavobacteriales and a Gammaproteobacterium in some Phenacoccinae, but the metabolic functions conferred by these symbionts remain unknown (Gruwell *et al* 2007, Gruwell *et al* 2010, Rosenblueth *et al* 2012).

Armored scale insects (Diaspididae) are associated with the obligate endosymbiont '*Candidatus* Uzinura diaspidicola' (Sabree *et al* 2013). The *Uzinura* symbiont belongs to Flavobacteria (phylum Bacteroidetes), making it distantly related to other known sternorrhynchan symbionts (Gruwell *et al* 2007). *Uzinura* has an extremely reduced genome but retains all the necessary genes for the synthesis of essential amino acids and

nitrogen recycling (Subandiyah *et al* 2000). The insect taxa belonging to this family are known to feed on the photosynthetic cells (mesophyll), rather than phloem sap as in most other Sternorrhyncha. In addition to Diaspididae, the flavobacterial symbionts have been characterized in multiple families of scale insects such as Lecanodiaspididae, Coccidae, Eriococcidae, Pseudococcidae, Ortheziidae, Coelostomidiidae, and Monophlebidae (Rosenblueth *et al* 2012). In most cases, the flavobacterial symbionts are accompanied by an Enterobacteriaceae symbiont (Gammaproteobacteria), and genomic analysis indicate that both symbionts contribute to nitrogen recycling and provisioning essential amino acids to their host (Rosas-Perez *et al* 2014, Rosenblueth *et al* 2012). Phylogenetic analyses based on the 16S rRNA genes reveal that all of the flavobacterial symbionts – with the exception of '*Candidatus* Brownia rhizoecola' (in Pseudococcidae: Phenacoccinae) – form a single monophyletic clade, implying an ancient infection event and subsequent codiversification with their hosts (Rosenblueth *et al* 2012).

5.5 SYMBIONT-ENABLED TRANSITION FROM PHLOEM TO XYLEM FEEDING IN AUCHENORRHYNCHA

Like most Sternorrhyncha, several groups of the Auchenorrhyncha are phloem feeders, e.g. planthoppers (Fulgoroidea: Cixiidae, Delphacidae, Flatidae, Fulgoridae), treehoppers (Membracoidea: Membracidae), and leafhoppers (Membracoidea: Cicadellidae) (Dietrich 2003). Most Auchenorrhyncha are engaged in an obligate dual symbiosis: '*Candidatus* Sulcia muelleri' (Bacteroidetes) synthesizes a set of seven or eight essential amino acids, and a co-primary symbiont, usually a Betaproteobacterium, synthesizes the two or three remaining essential amino acids (Bennett and Moran 2013). While *Sulcia* was retained in most host lineages since the origin of the symbiosis about 270 million years ago (Moran *et al* 2005), the betaproteobacterial symbiont has been repeatedly lost or replaced

(Buchner 1965, Koga *et al* 2013, McCutcheon and Moran 2010, Moran *et al* 2005). The clade consisting of '*Candidatus* Nasuia deltocephalinicola' in deltocephaline leafhoppers (Noda *et al* 2012), '*Candidatus* Zinderia insecticola' in spittlebugs (McCutcheon and Moran 2010), and '*Candidatus* Vidania fulgoroideae' in fulgorid planthoppers (Gonella *et al* 2011) likely represents the ancestral betaproteobacterial co-primary symbiont partner of *Sulcia* (Bennett and Moran 2013). This is supported by phylogenetic analyses revealing its codiversification with *Sulcia muelleri* and with its hosts since the origin of the symbiosis (Bennett and Moran 2013, Koga *et al* 2013, McCutcheon and Moran 2010, Moran *et al* 2005). Subsequent replacement events include the switch to '*Candidatus* Hodgkinia cicadicola' (Alphaproteobacteria) in cicadas (McCutcheon *et al* 2009), to *Sodalis*-like symbionts (Gammaproteobacteria) in philaenine spittlebugs (Koga *et al* 2013) and to '*Candidatus* Baumannia cicadellinicola' (Gammaproteobacteria) in some leafhopper taxa (Takiya *et al* 2006). These transitions in symbiotic syndromes may have played important roles for dietary and ecological shifts of the hosts. The acquisition of *Hodgkinia* and *Baumannia* symbionts, respectively, may have permitted cicadas and leafhoppers to transition from phloem to xylem sap. Both *Baumannia* and *Hodgkinia* can produce the same essential amino acids (methionine and histidine) that were produced by the ancestral symbiont they replaced. However, *Baumannia*'s comparatively large genome (686 kb) additionally encodes for pathways to produce vitamins and cofactors, which may have enabled leafhoppers to shift to xylem sap (Wu *et al* 2006), a resource that is even more nutritionally limiting than phloem sap (Redak *et al* 2004). Unlike *Baumannia*, *Hodgkinia* has a severely reduced genome (144 kb) that lost all its vitamin and cofactor biosynthesis pathways along with two genes involved in cystathione synthesis (metA and metB), an intermediate in methionine synthesis, and it contains a cobalamin biosynthesis genes (MetH) instead of MetE (cobalamin-independent

methionine synthase) in the last step of methionine synthesis unlike other related hemipteran symbionts indicating that the cicada and its symbionts have access to these compounds from the root xylem exuades inaccessible to their closely related insects (McCutcheon *et al* 2009). Additionally, symbiont replacements or losses have also occurred in phloem-feeding Auchenorrhyncha. Both *Sulcia* and *Nasuia* have been lost in *Scaphoideus titanus* (Deltocephalinae) instead a transovarially transmitted *Cardinium* (Bacteroidetes) plus a yeast-like symbiont related to those found in certain lineages of planthoppers and aphids have been acquired (Sacchi *et al* 2008). Some leafhopper groups like the Typhlocybinae (Cicadellidae) switched their diet from phloem to the more nutritious parenchyma plant tissue and have reportedly lost the dual symbionts (Buchner 1965, Moran *et al* 2005). However, the nutritional aspects of these cases have not been investigated yet.

5.6 ANCESTRAL SYMBIONT-ENABLED EXPLOITATION OF BRYOPHYTE HOST PLANTS BY COLEORRHYNCHA

The moss bugs (suborder Coleorrhyncha) are associated with the intracellular symbiont '*Candidatus* Evansia muelleri' (Gammaproteobacteria) that is closely related to *Carsonella* and *Portiera*, the primary endosymbionts of psyllids (Hemiptera: Sternorrhyncha: Psyllidae) and whiteflies (Hemiptera: Sternorrhyncha: Aleyrodidae), respectively (Kuechler *et al* 2013, Santos-Garcia *et al* 2014). However, phylogenomic analyses indicate that – despite the common ancestry of *Evansia, Carsonella*, and *Portiera* – the moss bug symbionts likely represent an independent infection event (cite 58 here). In addition to supplementing essential amino acids, *Evansia* synthesizes cofactors and is involved in sulphur metabolism to compensate for the low levels of

sulphur and nitrogen found in the moss bugs diet, the plant sap of mosses and liverworts (bryophytes) (Santos-Garcia *et al* 2014).

5.7 FREQUENT EVOLUTIONARY TRANSITIONS BETWEEN SYMBIOTIC SYNDROMES IN HETEROPTERA

The true bugs (suborder Heteroptera) consist of around 40,000 species and is the largest suborder of the Hemipteran order. Five of the seven infraorders (Dipsocoromorpha, Enicocephalomorpha, Gerromorpha, Leptopodomorpha, and Nepomorpha) are almost exclusively predatory and lack any known nutritional symbionts (Schaefer and Panizzi 2000). By contrast, symbiotic associations have been identified in the two most diverse infraorders Cimicomorpha and Pentatomomorpha (Buchner 1965, Glasgow 1914). In the Cimicomorpha, blood-sucking species of the families Reduviidae (assassin bugs) and Cimicidae (bedbugs) harbor symbiotic *Rhodococcus* (Actinobacteria) in the gut cavity (Ben-Yakir 1987), or *Wolbachia* (Alphaproteobacteria) in specialized bacteriomes (Hosokawa *et al* 2010), respectively. Although belonging to different bacterial phyla and being localized in different host tissues, both symbionts complement the hosts' diet with B vitamins that are deficient in vertebrate blood (Ben-Yakir 1987, Hosokawa *et al* 2010). The largest family of Cimicomorpha, the Miridae, exhibits a broad range of feeding habits including herbivory, carnivory, and omnivory (Wheeler 2001). Microbial associates have so far only been characterized in two mirid species, *Macrolophus pygmaeus* and *Nesidiocoris tenuis*, which are predominantly predatory species commonly used for biological control of agriculturally important crop pests (Caspi-Fluger *et al* 2014, Machtelinckx *et al* 2012). However, in addition to being an effective natural enemy of pest insects, they also have the ability to feed on the crop plants themselves. In these mirid bugs, the widespread reproductive manipulators *Wolbachia* and *Rickettsia* have

been identified in the gut cavity as well in the ovaries (Caspi-Fluger *et al* 2014, Machtelinckx *et al* 2012). Under lab rearing conditions, reproductive manipulation was not observed in *N. tenuis* (Caspi-Fluger *et al* 2014), raising the possibility of a nutritional role for their host when switching to a plant-based diet, although this needs to be substantiated by experimental evidence.

In the infraorder Pentatomomorpha, almost all members are phytophagous (mostly seed feeders), and most harbor symbiotic bacteria in the gastrointestinal tract or – less commonly – in bacteriomes. The specialized gastric ceaca or crypts in the distal part of the midgut harboring extracellular proteobacterial symbionts appear to be the ancestral symbiont-bearing structures in Pentatomomorpha (Glasgow 1914, Miyamoto 1961). While little is known about symbiotic bacteria in the ancestral superfamily Aradoidea, most species in the superfamily Pentatomoidea have midgut crypts that are inhabited by one of several distinct lineages of vertically transmitted Gammaproteobacteria (Abe *et al* 1995, Hosokawa *et al* 2005, Kikuchi *et al* 2009, Prado *et al* 2006, Schorr 1957). Bugs from the family Plataspidae (Heteroptera; Pentatomoidea) harbor the crypt-associated symbiont '*Candidatus* Ishikawaella capsulata' (Gammaproteobacteria). This symbiont exhibits a reduced genome that encodes for the biosynthesis of essential amino acids and vitamins, and in addition contains a plasmid encoding an oxalate decarboxylase gene, which may provide defense to the host against the widespread plant toxin oxalic acid (Hosokawa *et al* 2006, Nikoh *et al* 2011). Similarly, another pentatomoid family Urostylididae harbor gammabacterial symbiont ("*Candidatus* Tachikawaea gelatinosa"). This symbiont also possesses biosynthetic capabilities for provisioning essential amino acids and vitamins to the host (Kaiwa *et al* 2014). In addition, the experimental removal of the symbionts in several other closely related pentatomoid families resulted in high mortality and reduced growth (Abe *et al* 1995, Fukatsu and Hosokawa 2002, Kikuchi *et*

al 2009, Tada *et al* 2011). Overall this indicates that the symbionts associated with the bugs from pentatomoid superfamily are beneficial and could play an important nutritional role to their insect host.

Recent studies on the symbionts of the superfamilies Coreoidea, Lygaeoidea, and Pyrrhocoroidea indicate that this large monophyletic clade of bugs ancestrally harbored *Burkholderia* symbionts in midgut crypts, which were secondarily lost or replaced by other symbiotic syndromes (Kikuchi *et al* 2011a, Sudakaran *et al*., submitted). The symbiotic *Burkholderia* are phylogenetically diverse, and in some cases there is experimental evidence demonstrating horizontal acquisition from the environment in every generation, indicating a high degree of flexibility in the symbiosis (Kikuchi *et al* 2007, Kikuchi *et al* 2011b). Even though little is known about the function of *Burkholderia* symbionts in bugs, one report implicates the bacteria in insecticide resistance of the host, the bean bug *Riptortus pedestris* (Kikuchi *et al* 2012). When this insect was exposed to the organophosphate fenitrothion in the field, environmental *Burkholderia* strains resistant to this insecticide were acquired by the bugs and conferred protection to the host (Kikuchi *et al* 2012). As fenitrothion detoxification cannot be the ancestral benefit conferred by *Burkholderia* symbionts to their hosts, future studies addressing potential benefits through nutritional supplementation or detoxification of plant secondary metabolites are necessary to understand the ecological implications of the widespread association of bugs with these betaproteobacterial symbionts. Likewise, functional analyses of symbiont-provided benefits are urgently needed to understand the ecological factors underlying the repeated changes in symbiotic syndromes from crypt-associated *Burkholderia* to gammaproteobacterial symbionts localized in bacteriomes or midgut epithelial cells in several lygaeoid families (Kuechler *et al* 2011, Kuechler *et al* 2012, Matsuura *et al* 2012, Fig. 1).

An evolutionary transition in symbiotic syndromes with probable ecological implications has been recently reported in the superfamily Pyrrhocoroidea (Sudakaran *et al*., submitted). While members of the family Largidae are associated with the ancestral crypt-associated *Burkholderia* symbionts bugs in the sister family Pyrrhocoridae lost the crypts. Instead, they harbor a stable bacterial consortium made up of two Actinobacteria (*Coriobacterium glomerans* and *Gordonibacter* sp.), one Firmicute (*Clostridium* sp.), and one Gammaproteobacterium (*Klebsiella* sp.) localized in the lumen of the anoxic M3 region of the midgut (Salem *et al* 2013, Sudakaran *et al* 2012, Sudakaran *et al*., submitted). The actinobacterial symbionts specifically were shown to play an important role for the insect host by supplementing limiting B vitamins. Furthermore, the microbiota may be involved in nitrogen metabolism, degradation of complex dietary components (specifically cellulose), and detoxification of noxious plant secondary metabolites, specifically cyclopropenoic fatty acids (CPFAs) (Salem *et al* 2013, Salem *et al* 2014). These symbiont-provided benefits probably enable their insect hosts to successfully exploit a nutritionally inadequate food source (the seeds of Malvales plants) that is inaccessible to many other insects, due to the low concentrations of accessible B vitamins and the presence of toxic CPFAs. Even though seed feeding is widespread among pentatomomorphan bugs, the transition of the symbiotic syndrome to an anaerobic midgut microbiota appears to be confined to the Pyrrhocoridae (Sudakaran *et al.,* submitted). The specialized nutritional challenges associated with the dietary switch to Malvales seeds may have driven this evolutionary transition in symbiotic syndromes, which is supported by age estimations revealing an origin of the Pyrrhocoridae symbiosis coinciding with the evolution of the Malvales plant order (Sudakaran *et al.,* submitted).

5.8 ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS OF TRANSITIONS IN SYMBIOTIC SYNDROMES

The hemipteran insect order is one of the most extensively studied in terms of their symbiotic relationship with microorganisms. Members of Hemiptera are associated with a wide array of symbiotic syndromes from obligate intracellular symbioses associated with Sternorrhyncha, Auchenorrhyncha and Coleorrhyncha suborders that feed on plant sap (xylem and phloem) (Baumann 2005b) to extracellular symbioses housed in specialized structures such as crypts (Kikuchi *et al* 2011a) or in the gut lumen in seed feeding Heteropteran suborder (Salem *et al* 2013, Sudakaran *et al* 2012). In several cases, the Phylogenetic analysis have revealed a long term codiversification between certain insect groups and its bacterial symbionts dating to the origin of the insect group itself and in some cases even to the earliest stages of terrestrial herbivory on Permian vascular plants at least 270 million years ago (Moran 2007, Moran *et al* 2008). Despite the observed long term perseverance of symbiosis in hermpiteran order, several transitions in symbiotic syndromes with losses and replacements of primary symbionts, acquisitions of co-primary symbionts as well as changes in symbiont-bearing structures have arisen independently multiple times with several different bacterial groups including Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, and Gammaproteobacteria.

These numerous transitions in symbiotic syndromes observed within the hemipteran insect order can be explained by multiple mutually non-exclusive hypotheses. For instance, primary endosymbionts constantly face a plethora of evolutionary pressure bestowed by their entrapment within the host, the obligation to produce vitamins and nutrients in the face of unrelenting genome reduction and strong genetic drift. As a result such long term symbiotic relationship leads to not only massive genome reduction but

also an overall reduction in the quality of symbiont provided benefits. Thus, to compensate for the degradation in symbiont quality, the long term associate primary symbiont is often supplemented with an additional microbial symbiont as the symbiosis diversifies and if this additional symbiont can provide nutritional benefits to the insect host and is sufficiently common, easily accessible and/or stably transferred to every generation this leads to the eventual establishment of the additional symbiont as co-primary symbiont. Such a transition from single to dual symbiont state will result in the reduction of both symbionts genome ending in complete interorganism metabolic complementarity (McCutcheon *et al* 2009). In retrospect, this scenario explains the abundance of dual symbiosis observed across hemipteran order (Bennett and Moran 2013, McCutcheon *et al* 2009, Fig. 1). Alternatively, the ancestral primary symbiont could also be replaced altogether with a novel symbiont as observed in some cases within Hemiptera (Bennett and Moran 2013, Koga *et al* 2013, Koga and Moran 2014).

An additional advantage to the exposure of new symbionts is their ability to confer novel ecological trait to their insect host. This would enable to the host to either expand its ecological range or completely shift to novel niche. This is evident from the transitions in symbiotic syndromes witnessed in several hemipteran groups (Fig. 1). The multiple independent acquisitions of the obligate intracellular symbionts that can provision the essential amino acids facilitated the exploitation of the phloem sap by hemipteran insect taxa from the sub orders: Sternorrhyncha, Auchenorrhyncha, and Coleorrhyncha (Bennett and Moran 2013, Hansen and Moran 2014, Moran and Bennett 2014, Santos-Garcia *et al* 2014). In the case of leafhoppers, the transition from ancestral symbiosis to *Baumannia*, which possess the genomic potential to produce vitamins and cofactors in addition to essential aminoacids provided by the ancestral symbiont, enabled the switch from phloem feeding to xylem (Wu *et al* 2006). In the blood feeding Cimicomorphan bugs, the B

vitamin deficiency in the diet was compensated by the independent acquisition of vitamin supplementing symbiont (Reduviidae → Gut associated Rhododcoccus; Cimicidae → Bacteriome housed Wolbachia) (Ben-Yakir 1987, Hosokawa *et al* 2010). Lastly in the case of Pyrrhocoridae bugs their relationship with a gut associated symbiotic bacterial community that provide multilevel benefits such as breaking down complex food particles, provisioning vitamins and detoxifying plant toxins has led to their adaptation to feeding on malvales seeds (Salem *et al* 2013, Salem *et al* 2014, Sudakaran *et al* 2012, Sudakaran *et al*., submitted). The ecological relevance/implications of transitions are not known in several other cases. But even in cases of switching between host plants (but still utilizing the same type of food, e.g. phloem sap), such transitions could be relevant, since they might allow for coping with differences in nutrient composition or toxic compounds. This is supported by the rich diversity of symbiotic syndrome and the widespread presence of symbiosis across Hemiptera. This inherent ability of the hemipteran insects to be amenable to transit between diverse symbiotic syndromes has enabled their successful radiation into wide range of host plants.

5.9 CONCLUDING REMARKS

Microbes have played a major role in the facilitating herbivory in many different insect groups. They harbor an enormous range of metabolic capabilities that could provide a multitude of benefits to the insects. However the evolutionary and ecological cues responsible for the transition of the symbiotic syndrome in insects and the resulting ecological implication are less well understood. The high variety of symbiotic syndrome associated with hemipteran insects and their role in enabling the insect taxa to feed on the specific host plant makes it ideal system to address this question. So far most studies have focus on molecular analyses of symbiosis (phylogenetics, genomics), which have advanced our understanding a lot. But what's needed now are detailed studies on the nutritional ecology, in order to understand what a change in symbiosis and a change in host plant means for the host?, i.e. what are the nutritional compositions of the host plants? How can symbionts compensate for changes in the diet? How does a change in symbiotic syndrome affect host fitness?

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CHAPTER 6

SUMMARY

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SUMMARY

The gut microbial symbionts have had an enormous impact on the evolution of animals. These symbionts have been well documented to impart a vast repertoire of beneficial functions that significantly contribute to the fitness of their insect hosts ranging from provisioning nutrients, detoxifying plant toxins, exploiting novel food source, immunological priming, as well as protection against pathogens and parasites. Despite extensive studies on the functional aspect of the insect symbiosis research, in comparison there have been relatively few studies focused on the impact of microbial symbionts on the diversification and speciation of their insect host. In this doctoral thesis, the stability, specificity and functional importance of the gut microbiota of Pyrrhocoridae family bugs and the influence of the microbial symbionts on the exploitation of Malvales ecological niches and thereby resulting in the diversification of it insect host was investigated.

Stable and consistent gut microbial community of firebugs. The symbiotic microbial community associated with the midgut of the *Pyrrhocoris apterus* (Pyrrhocoridae) was comprehensively characterized using 454 16s amplicon pyrosequencing and cloning sequencing methods. This revealed that the symbiotic gut community is dominated by four specific bacterial species comprised of Actinobacteria *(Coriobacterium glomerans* and *Gordonibacter sp.*), Firmicutes (*Clostridium sp.*), and Proteobacteria (*Klebsiella sp.*). Further examination of the gut microbiota across the different developmental stages of the *P. apterus* revealed that the abundance of the core microbiota increased around the

development of the second nymphal stage of the firebugs coinciding with the start of feeding on their food source (linden seeds). This indicates that gut symbionts could possibly assist in breakdown of ingested particles to nutrients required by the host. Additionally gut symbiotic community composition was found to be qualitatively and quantitavely constant with the core microbiota being consistently abundant across *P. apterus* specimens collected from different geographical locations and diets.

Functional importance of the gut symbionts in firebugs. The combination of experimental manipulation with community analysis was used to analyze the functional importance of the gut symbionts to the firebugs. The experimental removal of the symbionts by egg surface sterilization resulted in significantly higher mortality and reduced growth rates in firebugs. Community level analyses by quantitative PCR of the core microbiota revealed that both the Actinobacterial symbionts (*C. glomerans* and *Gordonibacter sp.*) are vital for the fitness of the host.

Impact of the gut microbiota on the diversification of the Pyrrhocoridae bugs into malvales niche. The evolution of the Pyrrhocoridae-gut microbiota association was analyzed by reconstructing the host phylogeny and comprehensive characterization of the gut microbiota of different members of the Pyrrhocoridae family. The core microbiota identified in the *P. apterus* was widespread across the pyrrhocorid bugs. The insect taxa belonging to the sister family of Pyrrhocoridae, Largidae examined in this study revealed that they harbor crypt associated *Burkholderia* symbionts, which is similar to the symbiotic association found in closely related superfamilies Lygaeoidea and Coreoidea. This indicates that the association with *Burkholderia* symbionts is ancestral to all three super families Lygaeoidea, Coreoidea and Pyrrhocoroidea superfamilies. In the case of

Pyrrhocoridae family, the crypt associated symbiosis has been lost and instead bacterial community was acquired in their midgut region. The evolutionary origin of this association between the gut microbiota and the Pyrrhcoridae family coincides with the evolution of their host plant, Malvales. Overall this indicates that the acquisition of the gut microbiota enabled the Pyrrhocorid bugs to exploit a novel food source (Malvales) and thereby diversify into that niche.

CHAPTER 7

ZUSAMMENFASSUNG

Die mikrobielle Darmgemeinschaft hatte einen enormen Einfluss auf die Evolution der Tiere. Diese Symbionten können über ein breites Spektrum an nützlichen Funktionen die Fitness ihrer Wirtsinsekten erhöhen. Sie können etwa zusätzliche Nährstoffe bereitstellen, pflanzliche Gifte neutralisieren, helfen neue Nahrungsquellen zu erschließen , das Immunsystem stimulieren oder die Tiere auch gegen Parasiten und Krankheitserreger direkt verteidigen. Im Gegensatz zu umfangreichen Studien, die den funktionalen Aspekt von Insektensymbiosen behandeln, gibt es nur relativ wenige, die sich dem Einfluss mikrobieller Symbionten auf die Diversifizierung und Artbildung von Insekten widmen. In meiner Dissertation untersuchte ich die Stabilität und Spezifität sowie die funktionelle Bedeutung der Darmflora in der Familie der Feuerwanzen (Pyrrhocoridae). Des Weiteren untersuchte ich die Bedeutung der Darmflora für die Nutzung von Pflanzen der Ordnung Malvales als ökologische Nische und für die daraus resultierende Diversifizierung der Feuerwanzen.

Feuerwanzen weisen eine stabile, gleichbleibende Darmflora auf.

Die symbiotische Darmgemeinschaft, die mit dem Mitteldarm der Gemeinen Feuerwanze *Pyrrhocoris apterus* assoziiert ist, wurde mittels 454 16S Amplicon-Sequenzierung sowie Klonierung und Sanger-Sequenzierung charakterisiert. Die Darmgemeinschaft wird von vier Bakterienarten dominiert, den Aktinobakterien *Coriobacterium glomerans* und *Gordonibacter sp.* sowie *Clostridium sp.* (Frimicutes) und *Klebsiella sp.* (Proteobacteria). Weitere Untersuchungen zeigten, dass diese zentrale Gemeinschaft in der Entwicklung

der Feuerwanzen während des zweiten Nymphen-Stadiums etabliert wird, in dem die Nymphen beginnen, Lindensamen zu fressen. Das legt nahe, dass die Darmsymbionten daran beteiligt sind, die aufgenommene Nahrung für die Wirtsinsekten aufzuschließen. Die symbiotische Darmgemeinschaft ist außerdem in *P. apterus*-Individuen von verschiedenen Standorten, sowie von Individuen, die auf unterschiedlicher Nahrung gehalten wurden, qualitativ und quantitativ stabil.

Die Funktionelle Bedeutung der Darmsymbionten für Feuerwanzen.

Um die Funktion zu untersuchen, die die Darmsymbionten in Feuerwanzen erfüllen, verwendete ich eine Kombination aus experimenteller Manipulation und mikrobieller Gemeinschaftsanalyse. Die experimentelle Sterilisierung der Eioberfläche, über die die Symbionten an die Nachkommen weitergegeben werden, führte zu signifikant höherer Mortalität und niedrigeren Wachstumsraten während der Entwicklung der gemeinen Feuerwanze. Die Gemeinschaftsanalyse durch quantitative PCR der zentralen vier Bakterienarten zeigte, dass die beiden symbiotischen Aktinobakterien (*C. glomerans* und *Gordonibacter sp.*) für den Wirt lebensnotwendig sind.

Der Einfluss der Darmgemeinschaft auf die Diversifizierung der Pyrrhocoridae mit Malvales als primärer Futterpflanze.

Um Rückschlüsse über die Evolution der Darmgemeinschaft der Pyrrhocoridae zu ziehen, rekonstruierte ich die Phylogenie der Pyrrhocoridae und erstellte eine umfassende bakterielle Gemeinschaftsanalyse verschiedener Arten dieser Wanzenfamilie. Die zentrale Bakteriengemeinschaft, die ich bereits in *P. apterus* gefunden hatte, war in der gesamten Familie der Feuerwanzen weit verbreitet. Eine Analyse der Largidae, der Schwestergruppe der Pyrrhocoridae, zeigte, dass diese stattdessen Bakterien der Gattung *Burkholderia* in Darmkrypten beherbergen, die auch in den nah verwandten

Superfamilien Lygaeoidea und Coreoidea vorkommen. Dies weist darauf hin, dass die Symbiose mit *Burkholderia*-Bakterien den ursprünglichen Zustand in den drei Superfamilien Lygaeoidea, Coreoidea und Pyrrhocoroidea darstellt und die Wanzen der Familie Pyrrhocoridae diese Symbionten in Darmkrypten verloren haben und sich stattdessen die Symbiose mit einer anaeroben Bakteriengemeinschaft im Mitteldarm etabliert hat. Der evolutionäre Ursprung dieser symbiotischen Beziehung in der Familie der Feuerwanzen fällt zeitlich mit der Entstehung ihrer Futterpflanzen der Ordnung Malvales zusammen. Zusammenfassend deutet alles darauf hin, dass die Aufnahme dieser neuen mikrobiellen Darmgemeinschaft den Feuerwanzen ermöglichte, Malvengewächse als neue Nahrungsquelle zu nutzen und sich dadurch in dieser ökologischen Nische auszubreiten.

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DECLARATION OF INDEPENDENT ASSIGNMENT

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller-University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University, Jena or to any other university.

Jena, 21st January 2015

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